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Enzymology and inhibition of ghrelin acylation by ghrelin O-acyltransferase

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Abstract

Ghrelin is a circulating peptide hormone that plays a regulatory role in many physiological processes related to energy balance and metabolism. To be recognized by its receptor, growth hormone secretagogue receptor 1a (GHSR1a), the serine 3 residue of ghrelin must be octanoylated. This unique posttranslational modification is catalyzed by the enzyme ghrelin *O*-acyltransferase (GOAT), a topologically complex integral membrane protein. GOAT is an intriguing target for biochemical studies, as no other enzyme is known to catalyze octanoylation of a serine hydroxyl group. It is also an attractive target for diseases related to ghrelin signaling, such as diabetes and obesity, as blocking GOAT activity would decrease the levels of acylated (active) ghrelin. However, much about GOAT remains undefined: its catalytic residues, active site structure, and mechanism are unknown, and assays studying GOAT activity are limited. The lack of structural and mechanistic information regarding GOAT makes the rational design of small molecule inhibitors difficult, and the inability to easily apply GOAT activity assays to a high throughput format has limited the discovery of small molecule GOAT inhibitors. With these challenges in mind, we have made several improvements to GOAT activity assays which increase product stability and enable real-time monitoring of ghrelin acylation. A library screen of small molecules has revealed a novel class of small molecule inhibitors of human GOAT (hGOAT), which act by covalently modifying a functionally essential cysteine residue within the enzyme. Finally, mutagenesis studies have revealed several residues within hGOAT that are required for activity.

ENZYMOLOGY AND INHIBITION OF GHRELIN ACYLATION BY GHRELIN *O*-ACYLTRANSFERASE

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Table of Contents

List of Figures ix

List of Tables xi

List of Abbreviations xii

Chapter 1: Introduction

1.1 Protein post-translational modifications 1

1.2 Ghrelin 2

1.2.1 Discovery 2

1.2.2 Ghrelin maturation 3

1.2.3 Expression pattern 4

1.2.4 Physiology of ghrelin signaling 6

1.3 Ghrelin *O*-acyltransferase (GOAT) 10

1.3.1 Discovery of GOAT 10

1.3.2 GOAT substrate selectivity and recognition of ghrelin 13

1.4 Efforts toward targeting ghrelin signaling for therapeutic development 15

1.4.1 Rationale for ghrelin signaling as a therapeutic target 15

1.4.2 GOAT inhibitors (2008–2016) 15

1.5 GOAT activity assays – capabilities and challenges 21

1.5.1 Cell-based assays for GOAT activity 21

1.5.2 Microsomal assays employing radiography 23

1.5.3 Cat-ELCCA 24

1.5.4 GOAT activity assays employing fluorescently labeled peptides 26

1.6 Goals of this work 27

References for Chapter 1 28

Chapter 2: Ghrelin octanoylation is completely stabilized in biological samples by alkyl fluorophosphonates

2.1 Introduction 52

2.2 Results 55

2.3 Discussion and conclusions 65

2.4 Materials and methods 67

References for Chapter 2 74

Chapter 3: Identification and development of small molecule hGOAT inhibitors

3.1 Introduction 80

3.2 Results 83

3.3 Discussion and conclusions 102

3.4 Materials and methods 106

References for Chapter 3 118

Chapter 4: Mutagenesis studies to locate functionally essential residues within hGOAT

4.1 Introduction 130

4.2 Results 134

4.3 Discussion and conclusions 146

4.4 Materials and methods 149

References for Chapter 4 155

Chapter 5: Development of a fluorescence-based high-throughput assay for rapid identification of GOAT inhibitors

5.1 Introduction 158

5.2 Results 160

5.3 Discussion and conclusions 167

5.4 Materials and methods 170

References for Chapter 5 173

Chapter 6: Conclusions and future directions

6.1 Future inhibitor discovery and development 177

6.2 Developing tools to efficiently investigate GOAT enzymatic activity 179

6.3 Determination of GOAT substrate binding sites and catalytic mechanism 181

6.4 Summary and future directions 182

References for Chapter 6 185

Appendices

Appendix I: Reprint permission for reference 125, Chapter 1 190

Appendix II: Reprint permission for reference 140, Chapter 1 191

Appendix III: Reprint permission for reference 1, Chapter 2 196

Appendix IV: Reprint permission for reference 1, Chapter 3 199

Appendix IV: Curriculum vitae 200

List of Figures

Figure 1.1 Maturation of ghrelin.

Figure 1.2 Proposed topology of ghrelin *O*-acyltransferase within the ER membrane.

Figure 1.3 Acylated ghrelin mimics as GOAT inhibitors.

Figure 1.4 Structurally related lipidated small molecules as GOAT inhibitors

Figure 1.5 Structure of bisubstrate analog GO-CoA-Tat, a GOAT inhibitor that is active in cell- and animal-based models.

Figure 1.6 Cat-ELCCA format for measuring GOAT activity.

Figure 2.1 Proposed pathways for ghrelin deacylation in circulation and GOAT activity assays.

Figure 2.2 Alkyl fluorophosphonate inhibitor incorporation increases the yield of acylated peptide product in an in vitro GOAT acylation assay.

Figure 2.3 Octanoylated ghrelin mimetic peptide is protected from deacylation in the presence of MAFP.

Figure 2.4 Dependence of ghrelin peptide octanoylation on concentration of octanoyl CoA in the presence of MAFP.

Figure 2.5 MAFP treatment is compatible with acyl ghrelin measurement by ELISA.

Figure 2.6 MAFP treatment completely protects octanoylated ghrelin from deacylation by mammalian cellular and blood esterases.

Figure 3.1 Screening format for identification of novel small molecule inhibitors of hGOAT.

Figure 3.2 Multiple CDDO derivatives effectively inhibit hGOAT.

Figure 3.3 Structure activity analysis reveals multiple pharmacophores contribute to inhibition of hGOAT by synthetic triterpenoids.

Figure 3.4 Inhibition of hGOAT by steroid derivatives.

Figure 3.5 Inhibition of hGOAT activity by cyclohexenone derivatives.

Figure 3.6 hGOAT inhibition profile supports the involvement of a catalytically essential cysteine residue.

Figure 3.7 hGOAT inhibition profile supports a covalent reversible mechanism of inhibition.

Figure 3.8 Acyl ghrelin levels are decreased by treatment with CDDO-EA and CDDO-Me in a cellular assay.

Figure 3.9 The mouse isoform of GOAT (mGOAT) is not as strongly inhibited by CDDO-EA as is hGOAT.

Figure 3.10 hGOAT and mGOAT exhibit dramatically different susceptibilities to inhibition by cysteine-modifying agents.

Figure 3.11 Clustal Omega alignment of hGOAT and mGOAT sequences.

Figure 3.12 Potential mechanism for hGOAT-catalyzed ghrelin octanoylation employing a cysteine acyl-enzyme intermediate.

Figure 3.13 ^{1}H and ^{13}C NMR spectra for compounds **9-11**.

Figure 4.1 Clustal Omega alignment of GOAT sequences from multiple species.

Figure 4.2 Expression analysis by Western blot for hGOAT mutant variants containing alanine and conservative mutations.

Figure 4.3 Activity screening of hGOAT alanine mutations probing for potential general bases.

Figure 4.4 Activity screening of hGOAT conservative mutations probing for potential general bases.

Figure 4.5 Activity screening of hGOAT mutant variants probing the role of cysteines within hGOAT.

Figure 4.6 Design and expression analysis of hGOAT variants targeting cysteines within topological subdomains.

Figure 4.7 Topological model of hGOAT highlighting functionally essential residues.

List of Tables

Table 4.1 Forward and reverse mutagenesis primers used to make the indicated mutations to alanine within hGOAT.

Table 4.2 Forward and reverse mutagenesis primers used to make the indicated mutations to structurally conserved residues within hGOAT.

List of Abbreviations

AcDan	Acrylodan
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
AgRP	Agouti-related peptide
AMPK	Adenosine monophosphate-activated protein kinase
CDDO	2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid
CHO	Chinese hamster ovary
CoA	Coenzyme A
Dap	(*S*)-2,3-diaminopropionic acid
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GH	Growth hormone
GHSR1a	Growth hormone secretagogue receptor type 1a
GOAT	Ghrelin *O*-acyltransferase
GPI	Glycosylphosphatidylinositol
HDFP	Hexadecylfluorophosphonate
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGOAT	human ghrelin *O*-acyltransferase
Hhat	Hedgehog acyltransferase
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IC_{50}	Inhibitor concentration at which activity is reduced by half
LB	Lysogeny broth

LD_{50}	Lethal dose at which 50% of a sample is killed
MAFP	Methoxy arachidonyl fluorophosphonate
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MBOAT	Membrane-bound *O*-acyltransferase
mGOAT	Mouse ghrelin *O*-acyltransferase
mRNA	Messenger ribonucleic acid
NCC	NIH Clinical Collection
NEM	*N*-ethylmaleimide
NIH DTP	National Institutes of Health Developmental Therapeutics Program
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
PCR	Polymerase chain reaction
PORCN	Porcupine
RNA	Ribonucleic acid
RP-HPLC	Reverse phase high performance liquid chromatography
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS – polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
WT	Wild type

Chapter 1: Introduction

1.1 Protein post-translational modification

Protein biochemistry illustrates the tremendous impact and power of chemical diversity to construct and control biological systems. The first level of this diversity lies in the amino acid sequence of a protein, with the twenty common amino acid side chains presenting an array of chemical functionality. After translation in the ribosome, many proteins undergo post-translational modifications. These relatively small chemical modifications greatly expand the proteome, providing alternate structures, localization patterns, protein-protein interactions, and recognition motifs to proteins with identical amino acid sequences.[1]

One class of post-translational modification is lipidation, in which a hydrophobic carbon chain is attached to a protein. This one class of modifications is remarkably diverse, though a central theme in the role of lipidation is the facilitation of protein-membrane or protein-protein interactions. Some examples of lipidation include prenylation, GPI-anchor modification, and acylation. Protein prenylation is the attachment of a 15- or 20-carbon isoprenoid to a cysteine residue within a C-terminal Ca_1a_2X motif through an irreversible thioether bond. In the cases of canonical prenylated proteins such as the GTPase Ras proteins, prenylation drives membrane localization and protein-protein interactions.[2] Glycosylphosphatidylinositol (GPI)-anchored proteins are modified at their C-terminus by a complex GPI structure, containing a glycan core consisting of several modified mannose moieties and a phospholipid tail. This lipid modification enables localization to the plasma membrane, where the protein can carry out a wide range of physiological processes.[3]

Acylation, the attachment of a carbon chain through an amide, ester, or thioester bond, is itself still a broad category of post-translational modifications. Two of the most extensively studied and ubiquitous forms of acylation within the cell are *N*-myristoylation, the addition of a 14-carbon acyl chain to the amine of an N-terminal glycine, and *S*-palmitoylation, the addition of a 16-carbon acyl chain to a cysteine thiol, both of which typically promote trafficking of proteins to membranes.[4, 5] *O*-Acylation, the acylation of a serine or threonine hydroxyl, is less common in proteins than either *N*- or *S*-acylation.[5] *O*- and *S*-acyl modifications are readily reversed through cleavage of the ester or thioester bonds by esterases and thioesterases.[6-8] In the case of *S*-palmitoylation, this enables dynamic switching between functionally distinct forms of the proteins.[9] The amide bond of *N*-myristoylation is much more stable, and this modification is essentially irreversible during a protein's lifetime.[5]

1.2 Ghrelin

1.2.1 Discovery

The peptide hormone ghrelin is one of the many lipidated proteins within the eukaryotic proteome. However, its particular lipid modification is unique as is its role in endocrine signaling. Ghrelin was discovered in 1999 as the endogenous ligand for growth hormone secretagogue receptor 1a (GHSR1a).[10] During its initial discovery and characterization, synthetic ghrelin, consisting of the 28 amino acids encoded by its DNA, exhibited a shorter retention time when analyzed by reverse phase-HPLC compared to natural ghrelin isolated from rat stomach extracts, indicating that the natural hormone was modified by some hydrophobic moiety. Mass spectrometry analysis revealed that ghrelin's serine 3 hydroxyl was modified by *n*-octanoyl group. Acylation at the hydroxyl of serine, threonine, or tyrosine is relatively rare. No other proteins have

been shown to contain an *O*-octanoyl serine modification,[11] and acylation at the hydroxyl of serine, threonine, or tyrosine in general is rare. Only a handful of proteins bearing this class of modification have been identified: the signaling protein Wnt is acylated at a conserved serine residue by palmitoleic acid,[12] histone H4 protein is palmitoylated at a serine residue,[13] and two toxins found in spider venom, δ/ω-plectotoxin-Pt1a[14] and PLTX-II,[15] are palmitoylated at a serine and threonine residue, respectively. Ghrelin's serine octanoylation modification is absolutely essential for its ability to bind and activate the GHSR1a receptor,[10] even though both the acylated and unacylated form of the hormone are present in the bloodstream.[16]

Despite being unable to activate the GHSR1a receptor, researchers have found evidence that unacylated ghrelin plays a regulatory role of its own.[17] As will be discussed below, administration of unacylated ghrelin has been shown to antagonize several physiological effects of ghrelin.[18-20] However, it does not act as a direct antagonist of ghrelin binding to the GHSR1a receptor.[21]

1.2.2 Ghrelin maturation

Ghrelin must undergo a series of post-translational processing steps in order for it to reach its mature form (Figure 1.1). Ghrelin is first translated as a 117-amino acid precursor hormone, preproghrelin.[10] The N-terminal signal sequence is cleaved co-translationally to yield the 94-amino acid proghrelin, with co-translational trafficking to the lumen of the endoplasmic reticulum. Here, proghrelin is octanoylated at its serine 3 residue by the enzyme ghrelin *O*-acyltransferase (GOAT)[22,23] and is packaged into secretory granules. Proghrelin is subsequently cleaved by prohormone convertase 1/3[24] to produce the 28-amino acid mature form of ghrelin, which can then be released (along with the 28-amino acid unacylated ghrelin) into the bloodstream. Another

peptide hormone, obestatin, is an alternative cleavage product of proghrelin, consisting of 23 residues within the C-terminus of proghrelin.[25]

1.2.3 Expression pattern

Ghrelin is primarily expressed in the stomach.[10, 26] *In situ* hybridization, immunohistological staining, and radioimmunoassays identified both acylated and unacylated forms of ghrelin within distinct endocrine cells in the stomach (X/A-like cells in mice and rats, P/D_1-like cells in humans).[27, 28] Significant levels of both forms are found throughout the gastrointestinal tract, though at lower levels than in the stomach.[16, 29, 30] Ghrelin is also produced in significant levels in pancreatic islets, as determined by immunohistological staining,[27] though there isn't consensus regarding which type of islet cell(s) express the hormone.[31, 32] RT-PCR analyses have also revealed levels of ghrelin expression in the arcuate nucleus of the hypothalamus in rats and mice,[33] and pituitary tissue of rats and humans,[34, 35] both regions which express high levels of ghrelin's receptor.[36, 37] Low levels of ghrelin expression have also been detected in the other peripheral tissues such as kidney,[38] lung, gall bladder, prostate, and liver.[39]

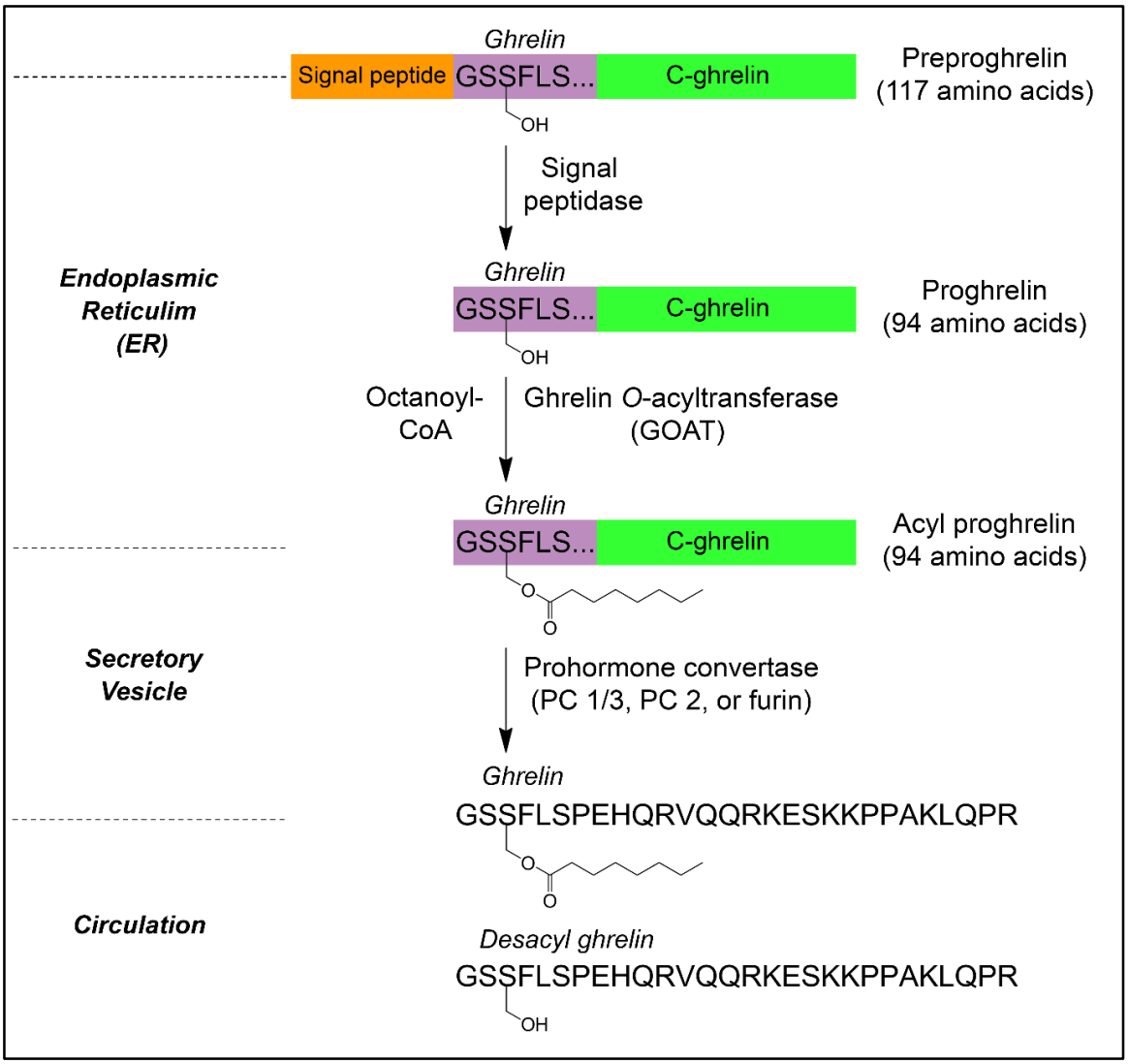

Figure 1.1 Maturation of ghrelin. Ghrelin is octanoylated at its serine 3 residue in the endoplasmic reticulum by ghrelin *O*-acyltransferase and using octanoyl CoA as the acyl donor.

GOAT is also most highly expressed in the stomach and pancreas, and significantly but to a lesser degree downstream of the stomach in the gastrointestinal tract.[22, 23] Specifically, *in situ* hybridization demonstrated colocalization of GOAT mRNA and ghrelin in the endocrine cells of the gastric mucosa of the stomach.[40] Significant but lower levels of GOAT expression are seen in the pituitary and hypothalamus, mirroring the hierarchy of ghrelin expression in the same tissues.[41]

1.2.4 Physiology of ghrelin signaling

Since its discovery, ghrelin has been shown to play a role in many physiological processes, many of which involve aspects of energy balance and metabolism.

Growth hormone release

As a ligand for the GHSR1a receptor, acylated ghrelin (hereafter referred to as ghrelin) stimulates release of growth hormone (GH) through an increase in intracellular Ca^{2+}.[10] In both rats and humans, intravenous administration of ghrelin results in an acute increase in GH concentration, with a peak within 15 minutes after administration and a return to basal levels after 1-2 hours.[42, 43] Ghrelin mimetics that activate the GHSR1a pathway have been investigated as potential therapeutics for age-related declines in physical strength resulting from decreased GH secretion.[44, 45] GOAT-knockout mice exhibit decreased GH release,[46] leading to lethal hypoglycemia under starvation conditions.[47] However, under normal diet conditions, GOAT-knockout mice display normal body weight and fat composition,[48] and ghrelin-knockout mice do not show growth abnormalities,[49, 50] indicating the ghrelin-growth hormone pathway may be at least partially redundant with other endocrine mechanisms regulating growth hormone secretion.

Appetite

Ghrelin is perhaps most well-known for its role in hunger signaling. Ghrelin remains the only known circulating hormone that increases the hunger sensation and food intake. Ghrelin binds to GHSR1a in the arcuate nucleus of the hypothalamus, stimulating neuropeptide Y (NPY) and Agouti-related peptide (AgRP) activity through the AMPK pathway in these neurons which regulate food intake and satiety.[33, 51] Administration of ghrelin results in an increase in food intake in rats,[52, 53] mice,[54] and humans[55] within minutes of ghrelin infusion. Endogenous ghrelin levels increase before meals, and decrease immediately after, independent of time- or food-related cues.[56-58] Fasting induces increased ghrelin expression in the stomach.[59]

Adiposity, obesity, and response to starvation

Increased food intake resulting from ghrelin activation of the arcuate nucleus can lead, unsurprisingly, to weight gain. However, increased ghrelin signaling can lead to increases in fat body mass through the upregulation of genes responsible for *de novo* lipogenesis in white adipose tissue, independent of food intake and energy expenditure.[60] These increases in body mass are also independent of GH and of NPY signaling in the hypothalamus, as ghrelin-induced adiposity was seen in NPY-deficient mice, and administration of GH alone did induce adiposity.[54] Ghrelin induces adiposity through the promotion of fatty acid storage and decreasing lipid metabolism.[54, 61] Specific adipogenic effects were seen with continuous infusion of ghrelin or unacylated ghrelin in bone marrow of GH-deficient rats.[62] A recent study discovered evidence for localization of GOAT within the plasma membrane of mouse bone marrow adipocytes, and that ghrelin-induced adiposity within bone marrow depended on the presence of GOAT.[63] This suggests that in bone

marrow adipocytes, the target cell mediates its own ghrelin signaling through direct activation of the hormone.

Ghrelin may play a role in protection from starvation. During severe calorie restriction, WT mice were able to maintain blood glucose levels, but glucose levels in GOAT knockout mice decreased to lethal concentrations.[47] While under normal conditions ghrelin promotes fat storage, during starvation, GH release stimulated by ghrelin can increase lipolysis in adipose tissue[64] and decrease the glucose used by muscle, or may directly mediate gluconeogenesis.[65]

Despite ghrelin's general positive correlation with food intake and adiposity, obese subjects exhibit lower levels of plasma ghrelin.[66-68] Exogenous ghrelin administration does not stimulate food intake, growth hormone secretion, or activation of NPY/AgRP in diet-induced obese mice,[69] and obese humans do not exhibit normal ghrelin decreases after meals.[70, 71] A potential exception to this observation is seen in patients with Prader-Willi syndrome. Patients with this genetic disorder exhibit symptoms such as mild mental retardation, growth hormone deficiency, short stature, and insatiable appetite (hyperphagia).[72, 73] Elevated ghrelin levels have been observed in cases of Prader-Willi syndrome,[74-77] although the mechanism by which ghrelin affects appetite in these patients has not yet been fully defined.[77-79]

Insulin regulation

Ghrelin inhibits glucose-stimulated insulin regulation.[80, 81] Endogenous ghrelin and insulin levels are normally inversely correlated throughout the day,[57] and exogenous administration of ghrelin suppresses insulin secretion and increases plasma glucose levels after meals.[80, 82]

Although unacylated ghrelin does not bind to GHSR1a and was initially thought to be an inactive ghrelin degradation product, administration of unacylated ghrelin has been found to

counteract the effects of ghrelin on insulin inhibition.[19, 83, 84] This indicates the ratio of ghrelin to unacylated ghrelin may be an important factor in insulin sensitivity.[85] Treatment of neonatal mice with ghrelin prevented the onset of diabetes,[86] and several studies have found that decreased ghrelin levels are associated with insulin resistance in both type I and type II diabetes in humans.[87-90] However it is unclear whether this is a direct cause or a compensatory effect.

An inhibitory role for unacylated ghrelin against insulin resistance is supported by the development of unacylated ghrelin analogs as therapeutics. Administration of AZP531, a cyclized peptide consisting of ghrelin's residues 6-13, improves insulin sensitivity in both mice and humans.[91, 92] As this compound lacks ghrelin's acylated N-terminus, it is not expected to bind to GHSR1a or GOAT,[10, 93, 94] suggesting that unacylated ghrelin and its ghrelin analogs exert their physiological effects via an undefined mechanism.

Anti-inflammation

Both ghrelin and its receptor are expressed in human T cells, and expression levels of both increase upon T cell activation.[95] Ghrelin was shown to inhibit production of multiple inflammatory cytokines in these and umbilical vein endothelial cells.[95, 96] Increased levels of circulating ghrelin are seen in patients with both chronic and acute inflammatory diseases such as bacterial endotoxin-induced inflammation,[97] ulcerative colitis,[98] and ankylosing spondylitis[99] in response to the inflammation, though lower levels have been seen in rheumatoid arthritis.[100]

Gastrointestinal motility

Soon after its initial discovery, ghrelin was shown to stimulate gastric acid secretion and gastrointestinal motility in rats.[101] These effects are blocked with either a GHSR1a antagonist or

anti-NPY antiserum, indicating that these effects are mediated though GHSR1a signaling and NPY activation.[102] Ghrelin appears to act both centrally and peripherally, as its promotility effects depend both on the vagus nerve[102, 103] and cholinergic neurons within the small intestine.[104] Low levels of circulating ghrelin are associated with gastritis.[105, 106] Ghrelin administration to rats with gastroesophageal reflux disease did not increase motility, indicating that ghrelin signaling may be impaired in some gastrointestinal diseases.[107]

Learning and memory

In addition to its many roles in energy homeostasis, ghrelin also has been associated with neurological functions. Ghrelin not only stimulates the physical sensation of hunger as described above, but also the motivation to seek and work for food through the reward system in the brain, mediated by the release of dopamine.[108-111] Ghrelin has been shown to be important in other reward-driven behavior, including the use of alcohol,[112, 113] nicotine,[114-116] and cocaine.[117]

Through the stimulation of the hippocampus and amygdala, ghrelin also facilitates learning and memory[111] through the promotion of long-term potentiation and increased neuron spine density.[118] It may enhance fear memory, such as that in posttraumatic stress disorder,[119] but may also protect against stress-induced symptoms of depression.[120]

1.3 Ghrelin *O*-acyltransferase (GOAT)

1.3.1 Discovery of GOAT

After ghrelin's discovery in 1999, the enzyme that catalyzes the octanoylation modification remained unknown until 2008 when it was discovered simultaneously by two independent research groups.[22, 23] Ghrelin *O*-acyltransferase (GOAT), belongs to the membrane-bound *O*-acyltransferase

(MBOAT) superfamily of enzymes. This enzyme family includes two other protein acyltransferases, hedgehog acyltransferase (Hhat) and Porcupine (PORCN).[121, 122] Other MBOAT enzymes, such as acyl CoA:cholesterol acyltransferases (ACATs)[123] and acyl CoA:diacylglycerol acyltransferase (DGATs)[124] modify small molecule substrates.

GOAT is a topologically complex integral membrane protein residing in the ER membrane and contains 11 transmembrane helices and one reentrant loop as determined by epitope tagging and selective permeabilization studies (Figure 1.2).[125] Surprisingly, this study found that residues N307 and H338, both of which are proposed to be involved in catalysis, reside on opposite sides of the membrane. Mutating either of these residues to alanine abrogates activity.[22] GOAT's active site is proposed to be within its C-terminal half, based on conservation with other MBOAT enzymes[22, 121] and photocrosslinking studies.[125] Beyond this, much about GOAT's structure and mechanism remain unknown, including specific residues directly involved in substrate binding and catalysis.

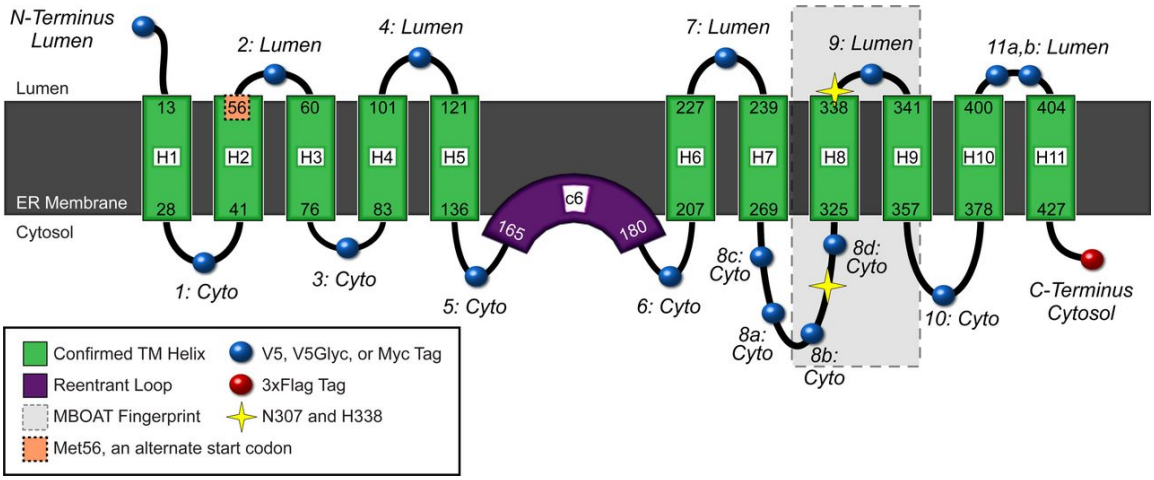

Figure 1.2 Proposed topology of ghrelin *O*-acyltransferase within the ER membrane, as reported by Taylor *et. al.*[125] Green bars represent transmembrane helices, and purple bar represents a reentrant loop. Functionally essential residues N307 and H338 are shown as yellow stars. Figure reproduced with permission from Reference 125 (Appendix I) © 2013 American Society for Biochemistry and Molecular Biology.

1.3.2 GOAT substrate selectivity and recognition of ghrelin

While the location and nature of the active site and catalytic mechanism of GOAT remain unknown, functional and structural elements within the ghrelin substrate which are important for recognition by GOAT have been determined. GOAT efficiently modifies short peptides consisting of ghrelin's first five residues (GSSFL-NH_2)[94, 126] and detectibly modifies substrates consisting of four residues (GSSF-NH_2).[126] GOAT exhibits a strong requirement for ghrelin's glycine 1 (G1) residue, with selectivity based both on charge and sterics. The mouse isoform of GOAT does not accept substrates with additional residues N-terminal to G1 of ghrelin.[94] Mutations of G1 which remove charge of the N-terminal amine, introduce steric bulk larger than one methyl group to the N-terminal amine, or incorporate any side chain are not tolerated.[94, 127, 128] Moderate mutations at ghrelin's S2 are tolerated in general; GOAT was found to modify a proghrelin substrate containing an S2A mutation as well as it did WT proghrelin,[94] and threonine was also accepted as well as serine at this position, suggesting the hydroxyl side chain may be recognized.[127] There may be steric and charge requirements at the S2 position, as substrates containing tryptophan, aspartate, or proline at this position were not reactive.[127, 128] GOAT displays a broad tolerance for mutations of F4, modifying substrates containing a wide range of natural and unnatural amino acids at this position with reduced but detectable activity,[94, 128] with the exceptions of lysine and glutamate which were completely unreactive.[128]

GOAT exhibits a strong selectivity for serine at the 3 position, the site of acylation. Mutation of S3 to alanine results in complete loss of reactivity,[22, 94, 127] indicating that GOAT will not octanoylate S2. Human GOAT will modify a threonine hydroxyl at this position, though less efficiently.[128] There is also evidence that GOAT will acylate an amine in place of the serine 3

hydroxyl.[129] These recognition and reactivity elements within ghrelin's N-terminus provided parameters for bioinformatics analysis to confirm that ghrelin is GOAT's only substrate.[128]

GOAT can accommodate a range of acyl-donor lengths. In their initial discovery of GOAT, Gutierrez *et. al.* transfected HEK293 cells with GOAT and preproghrelin, and supplemented the media with fatty acids of varying lengths. Through immunoprecipitation and analysis by MALDI-TOF, they detected ghrelin modified by acyl chains ranging in length from four to fourteen carbons.[23] An *in vitro* enzymatic assay using microsomal fraction containing GOAT, ghrelin peptide substrate, and acyl CoA acyl donors of different lengths was used to determine that GOAT modifies ghrelin with hexanoyl and octanoyl CoA more efficiently than with longer chain fatty acyl CoAs such as palmitoyl CoA.[126] This preference for medium chain fatty acids was also seen *in vivo:* when mice fed a diet rich in specific medium chain fatty acids or triglycerides (C6 – C10), the acyl modification of ghrelin isolated from their stomachs corresponded to the fatty acid ingested, and this correlation was not seen with ingestion of short chain (C4) or long chain (C16) fatty acids or triglycerides.[130]

To determine relative binding affinities of different length acyl chains to GOAT without additional complicating factors inherent in activity assays using different length acyl donors, Darling *et. al.* determined IC_{50} values for a series of peptide-based product mimetic inhibitors containing an acyl modification at the third residue linked through a hydrolytically stable amide bond. The structure-activity profile for this inhibitor series revealed that GOAT binds octanoyl chains much more strongly than other short or medium chains.[128]

In the absence of structural information regarding GOAT, these structure-activity parameters define recognition of both the ghrelin peptide and acyl CoA substrates by the enzyme, and begin to reveal what GOAT's active site could look like.[128] In addition, by determining which

functionalities within each substrate are most important for binding to the enzyme, we can incorporate those functionalities into GOAT inhibitors to maximize potency.

1.4 Efforts towards targeting ghrelin signaling for therapeutic development

1.4.1 Rationale for ghrelin signaling as a therapeutic target

Given ghrelin's role in diseases like diabetes, Prader-Willi Syndrome, and obesity, modulating the ghrelin pathway presents an attractive opportunity for the treatment of these diseases.[81, 131-134] There are numerous examples of GHSR1a antagonists and agonists in various stages of preclinical and clinical development.[131, 135, 136] An alternative approach is inhibition of GOAT-catalyzed acylation of ghrelin, rendering ghrelin unable to bind to and activate GHSR1a. As ghrelin is GOAT's only known and predicted substrate,[10, 22, 128] this route is expected to have a reduced probability for off-target effects.

As of 2012, few GOAT inhibitors had been reported in the scientific literature, and of those, only one was reported to inhibit GOAT in a cellular or organismal context.[94, 137, 138] Part of the difficulty in developing inhibitors lay in the lack of structural and mechanistic information available about the enzyme, as well as the limitations of assays for studying GOAT activity.

1.4.2 GOAT inhibitors (2008 – 2016)

Acylated ghrelin mimics as GOAT inhibitors

The majority of reported GOAT inhibitors are substrate or product analogs of ghrelin. The first GOAT inhibitors reported are [Dap^3]-ghrelin (1-5) and [Dap^3]-ghrelin (1-28), which feature the first five residues of ghrelin or the entire 28-amino acid sequence, respectively. In these peptide inhibitors, the serine 3 residue is replaced with (*S*)-2,3-diaminopropionic acid (Dap) in which the

side chain amine of this residue is octanoylated (Figure 1.3a).[94] Structure activity analysis of the acyl group length revealed that an octanoyl chain contributes to binding more strongly than any other length.[128] Both [Dap^3]-ghrelin (1-5) and (1-28) inhibit GOAT in an *in vitro* enzyme assay with IC_{50} values of 1 μM and 0.2 μM, respectively. However, as peptides these inhibitors have limited cell permeability and stability to degradation. Given their structural similarity to ghrelin, they may also activate GHSR1a, as the first four residues of ghrelin and a nonspecific hydrophobic group at S3 are sufficient to activate the receptor.[93]

To explore the potential for developing more cell permeable and biostable analogues of acylated ghrelin mimetic inhibitors, Zhao and workers developed GOAT inhibitors with triazole-linked acyl chain analogues.[139] These inhibitors were based on the GSSFL pentapeptide sequence present in the previously reported GOAT inhibitors,[94] with the incorporation of an azidoalanine at the third position in place of the serine residue that serves as the acylation site. This substitution enabled convenient modification of the peptide with a wide range of derivatives at this position through a copper-catalyzed Huisgen 1,3-dipolar cycloaddition between the azide and a substituted alkyne. Several derivatives of these peptides inhibited GOAT in an *in vitro* enzymatic assay, with the most potent featuring a phenylpropyl triazole modification (Figure 1.3b), inhibiting GOAT with an IC_{50} value of 0.7 μM. While these inhibitors face many of the same challenges to therapeutic viability as the [Dap^3]octanoyl-ghrelin inhibitors described above, they establish tolerance of the ghrelin N-terminus scaffold to chemical modification while maintaining potency. Thus, further modifications may be made to address the issues of cell permeability and GHSR1a activation.

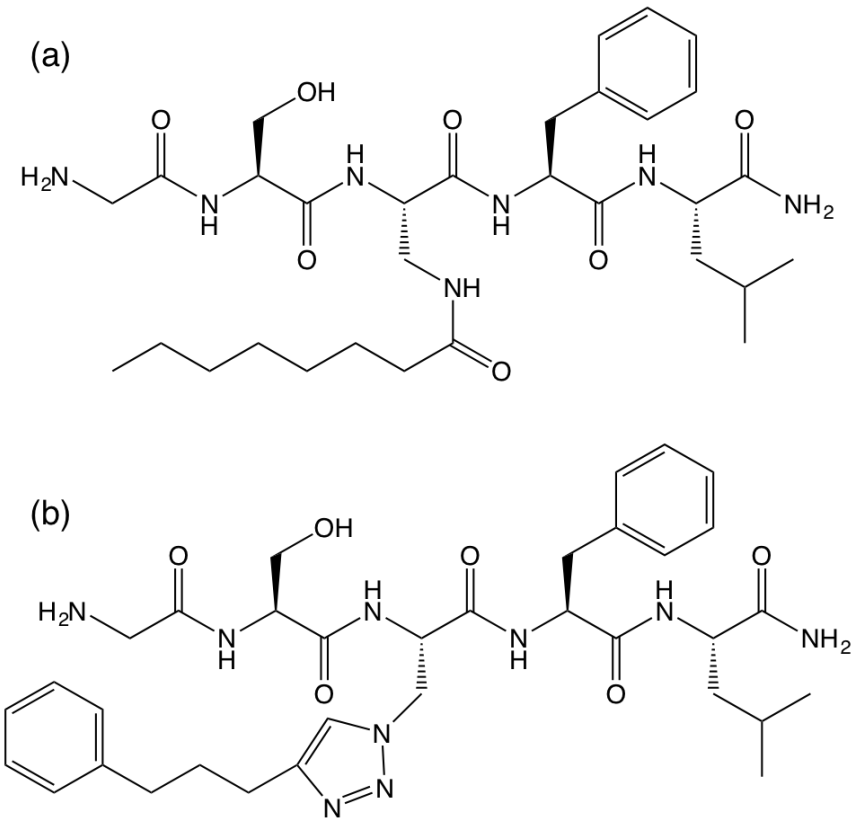

Figure 1.3 Acylated ghrelin mimics act as GOAT inhibitors. (a) Pentapeptide [Dap^3]-ghrelin (1-5)-NH_2 mimics the first five residues of ghrelin, with the ester linkage of octanoyl serine 3 replaced by a hydrolytically stable amide bond.[94] (b) Phenylpropyl triazole-linked ghrelin peptide mimics the first five residues of ghrelin, with the ester linkage of octanoyl serine 3 replaced by ta hydrolytically stable triazole formed by a Huisgen 1,3-dipolar cycloaddition between an azide and alkyne.[139]

Lipidated small molecules

In addition to the acylated ghrelin mimetic inhibitors described above, two small molecules have been reported to inhibit GOAT in an *in vitro* enzyme assay (Figure 1.4). These structurally-related inhibitors were identified from a library screen of small molecules and inhibit GOAT with IC_{50} values in the low micromolar range in an enzyme-based assay.[138, 140] While these compounds are more "drug-like" in their overall structure compared to the previously discussed peptide-based GOAT inhibitors, they have not been reported to be effective in cell-based or animal studies of GOAT inhibition.

GO-CoA-Tat

Validation of GOAT inhibition as a potential therapeutic avenue came from the development of GO-CoA-Tat, a bisubstrate analog GOAT inhibitor (Figure 1.5).[137] GO-CoA-Tat features the first ten residues of ghrelin, with an octanoyl coenzyme A analog attached to the third residue through a hydrolytically stable amide linkage, and an 11-residue HIV Tat-derived peptide attached to the C-terminus to enable cell permeability. Treatment with GO-CoA-Tat inhibits ghrelin acylation by GOAT in enzyme- and cell based assays as well as in mice. Administration of this inhibitor to mice improved glucose tolerance and reduced weight gain induced by high-fat diet.[137] Treatment with GO-CoA-Tat has also demonstrated effectiveness in decreasing food intake in freely-fed rats[141] and in attenuating food intake, food foraging, and food hoarding behavior in food-deprived Siberian hamsters.[142]

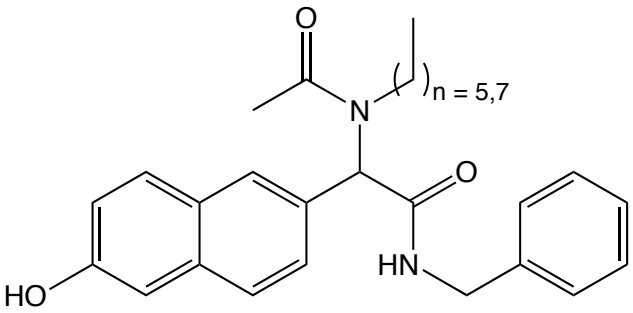

Figure 1.4 Structurally related lipidated small molecules as GOAT inhibitors. Compounds were identified by Garner and Janda through a screen of small molecules.[138]

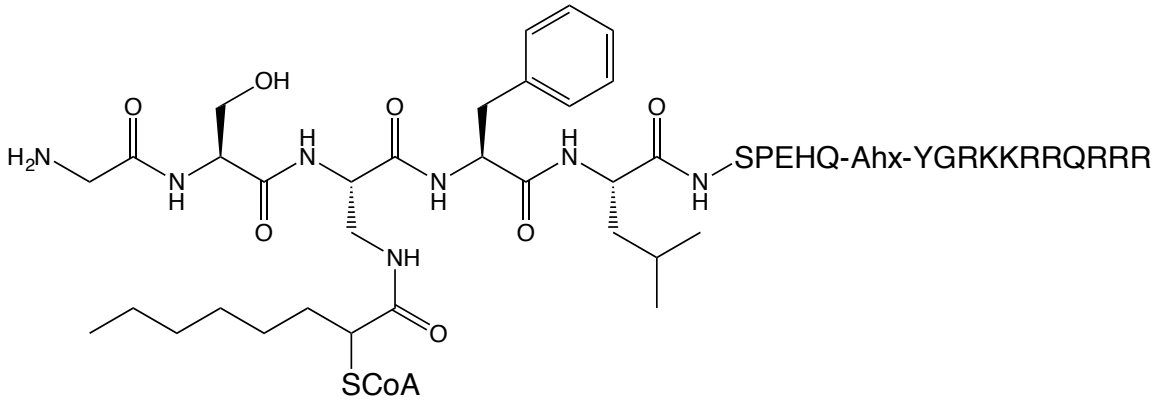

Figure 1.5 Structure of bisubstrate analog GO-CoA-Tat, a GOAT inhibitor that is active in cell- and animal-based models.[137, 141, 142] GO-CoA-Tat mimics the first ten residues of ghrelin, with a Tat peptide sequence (-YGRKKRRQRRR) appended to the C-terminus through an aminohexanoate (Ahx) linker to enable cell permeability. The serine 3 residue is replaced with octanamide Dap, with CoA covalently linked to the octanoate.

1.5 GOAT activity assays – capabilities and challenges

The lack of potent small molecule GOAT inhibitors prevents the validation of modulating ghrelin signaling as a therapeutic avenue. However, as the structure of the GOAT active site and nature of the GOAT catalytic mechanism remain unknown, the rational design of small molecule, "drug-like" inhibitors is not possible at this point. Screening large libraries of small molecules can allow identification of inhibitors as hit and lead compounds, but in the case of ghrelin octanoylation this approach is complicated by the limitations of current GOAT activity assays and the inability to adapt them to a high-throughput format.

Established assays for measuring ghrelin acylation by GOAT activity have either been cell-based, or have relied on microsomal fraction from cells expressing GOAT as an enzyme source *in vitro*.

1.5.1 Cell-based assays for GOAT activity

Several cells lines have been identified to study ghrelin acylation by GOAT. In 2001, human medullary thyroid carcinomas (human TT cells) were found to produce acyl ghrelin,[143] and several years later, Gutierrez *et. al.* used this cell line to identify GOAT as the acyltransferase that modifies ghrelin.[23] While levels of acyl ghrelin detected from these cells were low, supplementing the media with octanoic acid increased the amount detected by mass spectrometry.[23] Both acylated and unacylated forms of ghrelin were identified in a human erythroleukemia (HEL) cell line. Remarkably, ~90% of the ghrelin detected in the cells and in the media was acylated. Yang *et. al.*[22] transfected preproghrelin and candidate acyltransferases into rat insulinoma INS-1 cells to identify GOAT as the ghrelin acyltransferase, detecting acylated ghrelin by peptide separation on HPLC

followed by SDS-PAGE and immunoblotting with an anti-ghrelin antibody. They then used this cell line to transfect mutant forms of GOAT to identify functionally required residues.[22]

In 2010, Iwakura *et. al.* established a novel cell line, MGN3-1, isolated from the gastric tumor of a mouse.[145] These cells produced ~140-fold more total ghrelin and ~5,000-fold more acylated ghrelin compared to TT cells, as measured by N-terminal and C-terminal ghrelin radioimmunoassay. Zhao *et. al.* established the PG1 and SG1 cell lines from pancreatic and stomach tumors, respectively, of mice.[146] When PG1 cells were incubated with octanoic acid, significant levels of acylated ghrelin was detected in both the cells and in the medium, indicating that ghrelin was not only being produced but secreted as well. SG1 cells were used to determine that an analog of unacylated ghrelin inhibited secretion of acyl ghrelin.[147] Ghrelin and GOAT were also found to be expressed in cell lines derived from prostate cancer tissues (PC3 cells).[148, 149] Ghrelin (at concentrations up to 5 nM) increases PC3 cell proliferation by ~33%, providing a possible readout for the levels of ghrelin produced.[148] Treatment with ghrelin, but not unacylated ghrelin, downregulated expression of GOAT in these cells, which may complicate studies of GOAT activity.[149]

Both HeLa and HEK cell lines have also been used to study GOAT activity. Both have been stably transfected with genes for the human isoform of the ghrelin precursor preproghrelin and the mouse isoform of GOAT (mGOAT).[137] When supplemented with octanoic acid, acyl proghrelin is produced, and levels of both acyl and unacylated ghrelin can subsequently be quantified using an enzyme-linked immunosorbent assay (ELISA).

1.5.2 Microsomal assays employing radiography

GOAT activity can be measured in *in vitro* assays using microsomal fraction from mammalian or insect cells expressing recombinant GOAT. The first such microsomal assay was reported by Yang and coworkers,[94] in which baculovirus containing the gene for mouse GOAT (mGOAT) was utilized to express mGOAT in Sf9 insect cells. The crude microsomal protein fraction containing mGOAT was enriched through fractional ultracentrifugation, with this microsomal protein fraction shown to acylate purified proghrelin when incubated with radioactively labeled octanoyl CoA. Octanoylated proghrelin was detected by isolating proghrelin from the reaction mixture by affinity binding to a nickel resin using a C-terminal His_8 tag appended to proghrelin followed by scintillation counting of ^{3}H-labeled octanoate.

Barnett *et. al.* developed a variation of this assay, using microsomal fraction isolated from HEK293 cells and ghrelin(1-27) bearing a C-terminal biotin modification, with acylated ghrelin detected by scintillation counting of ^{3}H-labeled octanoate following streptavidin-pulldown of ghrelin peptides.[137] Subsequent studies reported by this group used microsomal fraction isolated from insect cells.[125, 129]

Ohgusu *et. al.* developed an *in vitro* GOAT activity assay employing microsomal fraction isolated from CHO cells expressing GOAT as the enzyme source and unacylated ghrelin and octanoyl CoA substrates. After incubation of all reaction components, acylated and total ghrelin were separated by RP-HPLC. Each fraction was analyzed by ELISA or radioimmunoassay, using anti-ghrelin(1-11) antiserum to detect octanoyl ghrelin, and anti-ghrelin(13-28) to detect total ghrelin.[126]

1.5.3 Cat-ELCCA

Garner and Janda[140] developed a GOAT activity assay using an enzyme-linked click-chemistry assay (cat-ELCCA) (Figure 1.6). In this assay, the first five amino acids of ghrelin (GSSFL) were immobilized on a streptavidin-coated plate using a C-terminal biotin group. Microsomal fraction containing mGOAT and octynoyl CoA were incubated in the plate, leading to acylation of the immobilized ghrelin peptide. Following GOAT-catalyzed octynoylation, azide-labeled horseradish peroxidase (HRP) was conjugated to the product via a copper-catalyzed Huisgen cycloaddition. The ghrelin-HRP conjugate could then be detected using a fluorogenic substrate which is activated in the presence of HRP and hydrogen peroxide. This assay enabled the discovery of two lipidated small molecule inhibitors of GOAT through a screen of a small library of compounds (Figure 1.4).[138]

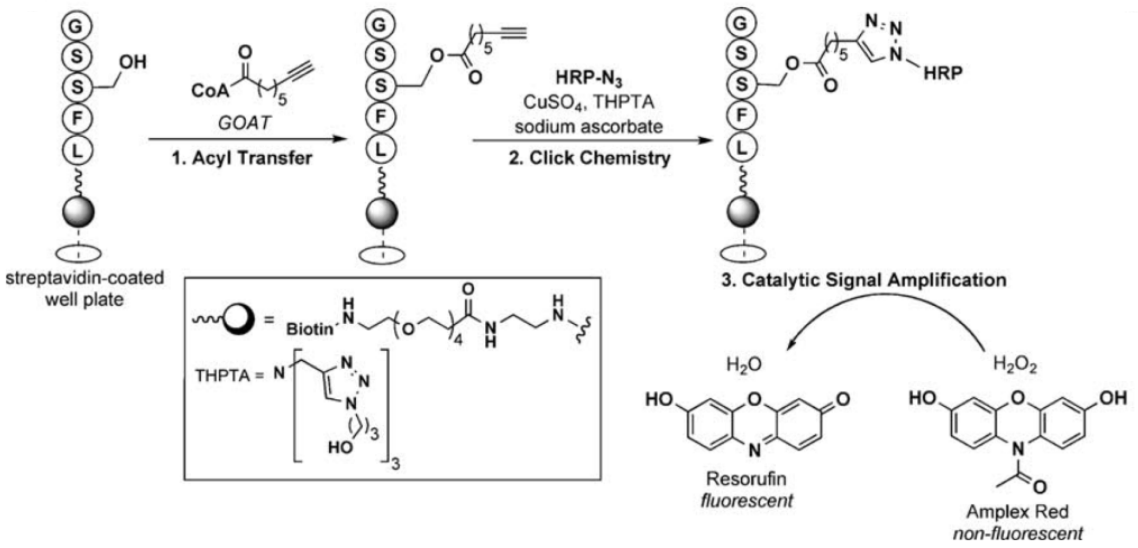

Figure 1.6 Cat-ELCCA format for measuring GOAT activity, as reported by Garner and Janda.[140] An N-terminal ghrelin peptide is immobilized onto a streptavidin-coated plate, and is modified by octynoate in the presence of GOAT. The alkyne of acylated ghrelin enables conjugation of an azide-functionalized horseradish peroxidase (HRP), which allows for activation of a profluorecent substrate in the presence of hydrogen peroxide. Figure reproduced with permission from reference 140 (Appendix II). © 2010 John Wiley and Sons, Inc.

1.5.4 GOAT activity assays employing fluorescently labeled peptides

To enable structure-activity analysis of ghrelin recognition by GOAT, our research group developed a microsomal GOAT activity assay using a fluorescently-labeled ghrelin peptide substrate.[127] A five-residue peptide based on the N-terminus of ghrelin was appended with a C-terminal cysteine (GSSFLC), which could be modified by acrylodan, a thiol-reactive fluorophore. After incubation with octanoyl CoA and microsomal fraction from insect cells infected with human GOAT (hGOAT) baculovirus, the fluorescent peptide is octanoylated. In contrast to previously reported GOAT assays, in which the detected label is on the acyl donor, the fluorophore being on the peptide substrate enables the simultaneous detection of both the unacylated and acylated forms of the peptide through their separation by reverse phase-HPLC while monitoring fluorescence of acrylodan. Octanoylation of the substrate increases its hydrophobicity, resulting in an increase in retention time.

Each of these reported *in vitro* assays has been limited by low levels of product conversion, often less than one percent of the total ghrelin/proghrelin substrate.[94, 125-127] It has been proposed that thioesterases within the microsomal protein fraction used for these assays have been at least partly responsible for this lack of reaction completion. To ameliorate these effects, many of the microsomal assays described above include long chain fatty acyl CoAs such as palmitoyl CoA. These long chain fatty acyl CoAs, which are not themselves substrates for GOAT-catalyzed acylation of ghrelin,[126, 130] compete for these contaminating thioesterases and limit the hydrolysis of octanoyl CoA, to varying degrees of success.[94, 127, 140] However, these assays are still limited in their ability to study GOAT activity, with conversion rates ranging between <1% to 10%, and endpoints reached within 5 minutes to 1 hour.

1.6 Goals of this work

The ever-growing number of physiological pathways in which ghrelin is reported to be involved necessitates thorough characterization of the enzyme that catalyzes its acylation. Without the ability to purify GOAT in its active form, the determination of its structure and mechanism remains elusive, which in turn limits the rational design of inhibitors. Despite these limitations, screening for inhibitors using our current assay is still a reasonable path toward the discovery of novel small molecule GOAT inhibitors, and valuable information about functionally essential residues within GOAT can be determined through site-directed mutagenesis.

Chapter 2 describes the application of alkylfluorophosphonate esterase inhibitors to *in vitro* GOAT activity assays to protect against product degradation. This development improves product conversion and the dynamic range of GOAT assays. In Chapter 3, the discovery of a new class of hGOAT inhibitors through a library screen of small molecules is described. Structure activity analysis of these inhibitors reveals they act through alkylation of a functionally required cysteine within hGOAT. Chapter 4 describes mutagenesis analysis of hGOAT, through which several functionally required residues within the enzyme are identified. A new assay based on fluorescence enhancement of a ghrelin substrate upon acylation is described in Chapter 5. This new assay is amenable to a high-throughput format, and it is applied to a primary screen of a small molecule library. These studies will further our understanding of how GOAT octanoylates ghrelin, and will progress the development of small molecule inhibitors of GOAT, which may lead toward therapeutics targeting ghrelin signaling.

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Chapter 2: Ghrelin octanoylation is completely stabilized in biological samples by alkyl fluorophosphonates

Portions of this chapter have been previously published and reprinted with permission from reference 1: McGovern-Gooch, K. R. Rodrigues, T., Darling, J. E., Sieburg, M. A., Abizaid, A. and Hougland, J. L. (2016), Ghrelin octanoylation is completely stabilized in biological samples by alkyl fluorophosphonates. *Endocrinology,* 157(11):4330-4338. Copyright 2016, Oxford University Press. T. Rodrigues performed experiments investigating ghrelin hydrolysis in rat blood. M. A. Sieburg performed experiments investigating ghrelin hydrolysis in HEK293FT cell lysate and MAFP compatibility with ELISA measurements of acyl ghrelin.

2.1 Introduction

The nature of GOAT as an integral membrane protein has prevented its purification or solubilization while maintaining enzyme activity. Thus, *in vitro* assays have relied on membrane protein fraction enriched from insect or mammalian cells expressing GOAT, as described in the previous chapter. Each of these assays, including the assay developed by our lab, have suffered from low levels of product conversion and incomplete endpoints.[2-5]

One possible explanation for the low endpoints observed is that octanoyl CoA is cleaved by thioesterases within the microsomal fraction. When measuring the amount of [^{3}H]octanoate generated from [^{3}H]octanoyl CoA when incubated with microsomal fraction, Yang *et. al.* found nearly all of the octanoyl CoA was hydrolyzed within 5 minutes.[2] In the presence of palmitoyl CoA, the majority of the octanoyl CoA was protected, indicating the long chain fatty acids compete for these thioesterases. Therefore, some GOAT activity assays incorporate palmitoyl CoA to limit the degradation of octanoyl CoA and increase octanoylated ghrelin product formation.[2, 4, 6-8] While this modification improved the observed octanoyl ghrelin formed in these reactions, the levels of product detected in these assays were still low (<1-10%). Assays that use higher concentrations of the octanoyl CoA acyl donor do not benefit from addition of palmitoyl CoA.[5]

Another possible explanation for the low levels of product conversion is the hydrolysis of the serine ester of the acyl ghrelin product itself. In biological studies, the ester linkage on serine 3 of acyl ghrelin has been shown to be susceptible to esterase-catalyzed hydrolysis within serum, tissue homogenates, and cell lysate,[9-14] and multiple esterases have been identified as able to hydrolyze ghrelin *in vitro*.[14-17] These studies demonstrate the esterase activity must be deactivated either by acidification or protease/esterase inhibitor treatment to stabilize acyl ghrelin for detection when collecting samples.[9-11, 15, 16, 18, 19] The ester linkage of the acyl peptide product in *in vitro*

assays may also be susceptible to contaminating esterases in the microsomal fraction (Figure 2.1), similar to the contaminating thioesterases responsible for hydrolyzing the acyl donor.[2]

To enhance the levels of acyl ghrelin product generated in our GOAT activity assay, the ability of potential esterase inhibitors to block product degradation was examined. Alkyl fluorophosphonates were found to inhibit esterases within microsomal protein fraction, cell lysate, and blood plasma, preventing the hydrolysis of the octanoyl modification. Incorporation of these esterase inhibitors enables the more accurate determination of GOAT activity *in vitro* and of true concentrations of acyl and unacylated ghrelin *in vivo*.

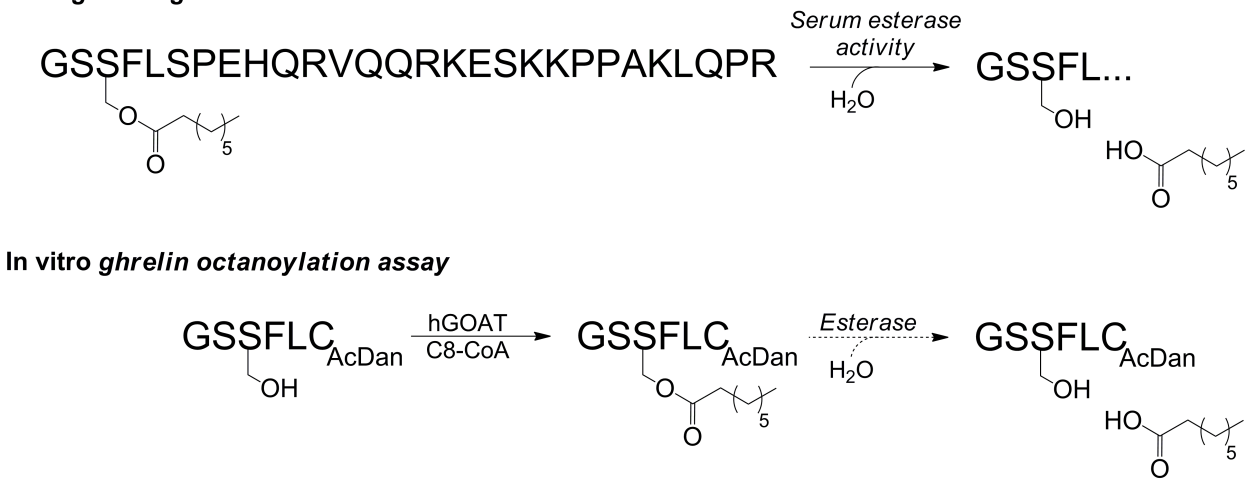

Figure 2.1 Proposed pathways for ghrelin deacylation in circulation and GOAT activity assays. Ghrelin in circulation is converted to unacylated ghrelin by serum esterase activity; esterase activity in microsomal protein preparation could lead to peptide deacylation after hGOAT-catalyzed octanoylation of a ghrelin mimetic peptide substrate. Reprinted with permission from reference 1. © 2016, Oxford University Press.

2.2 Results

Alkyl fluorophosphonate treatment increases the extent of ghrelin peptide octanoylation

Several potential esterase inhibitors were initially tested in our hGOAT activity assay. Both sulfonyl fluoride and fluorophosphonate-based compounds have been used to suppress ghrelin deacylation during sample work-up from biological sources.[9, 10, 15, 19] To test the ability of these classes of compounds to protect acyl ghrelin in the new context of a microsomal fraction-based GOAT activity assay, human GOAT (hGOAT)-containing microsomal fraction was preincubated with 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), hexadecyl fluorophosphonate (HDFP), or methoxy arachidonyl fluorophosphonate (MAFP) (Figure 2.2a) at 1 and 5 μM before initiating the octanoylation reaction with octanoyl CoA and fluorescently-labeled ghrelin peptide substrate, $GSSFLC_{AcDan}$. These reactions were incubated at room temperature for 3 hours, well beyond our previously observed reaction endpoint of 1 hour.[5] At this point in our standard assay, reduced concentration of octanoylated $GSSFLC_{AcDan}$ product is observed, potentially reflecting the deacylation by esterases outpacing the GOAT-catalyzed acylation in our proposed model. (Figure 2.1)

Relative to the vehicle control, addition of water-soluble sulfonyl fluoride AEBSF neither increased nor decreased the amount of observed octanoylated peptide. However, inclusion of either of the alkyl fluorophosphonate compounds, HDFP and MAFP, yielded a ~2-fold increase in the amount of acylated peptide compared to the DMSO-only control. (Figure 2.2b) The selectivity for enhancement by alkyl fluorophosphonates supports a model whereby deacylation of the ghrelin peptide is catalyzed by a membrane associated esterase or thioesterase.[20]

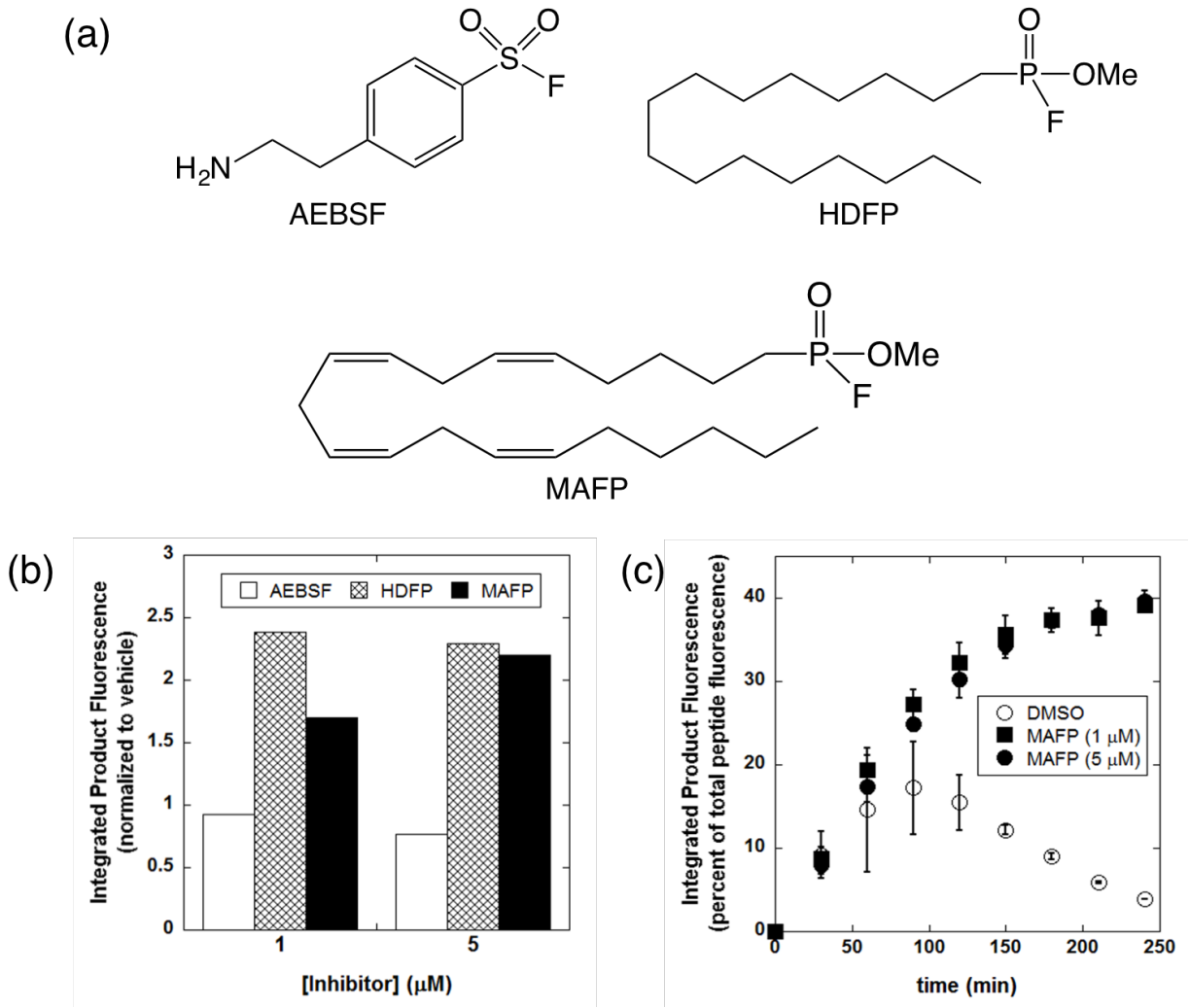

Figure 2.2 Alkyl fluorophosphonate inhibitor incorporation increases the yield of acylated peptide product in an *in vitro* GOAT acylation assay. (a) Structures of potential esterase inhibitors AEBSF, HDFP, and MAFP. (b) Treatment with the alkyl fluorophosphonate inhibitors HDFP and MAFP lead to increased levels of octanoylated peptide substrate after 3 hours of reaction, compared with the vehicle alone (not shown). Treatment with AEBSF did not yield increased product concentration compared with the vehicle control. (c) Time dependence of $GSSFLC_{AcDan}$ octanoylation in the presence and absence of MAFP. Open circles, treatment with DMSO (vehicle); filled squares, treatment with 1 μM MAFP; filled circles, treatment with 5 μM MAFP. Errors represent the standard deviation from a minimum of 3 independent trials. (b) and (c) reprinted with permission from reference 1.

To further characterize fluorophosphonate-mediated enhancement of ghrelin acylation, we examined the time-course of the hGOAT reaction in the presence of MAFP. (Figure 2.2c) In the absence of fluorophosphonate, the level of acyl ghrelin peptide fluorescence reaches a maximum of ~15% (relative to the total peptide fluorescence) around 90 minutes before decreasing to almost zero. In contrast, in the presence of MAFP, ghrelin peptide fluorescence continued to increase before reaching a maximum of ~40% of total peptide fluorescence after 3 hours and leveling off. The apparent velocity of both the untreated and MAFP-treated reactions is similar at early time points, indicating that the fluorophosphonates enhance product formation through protection of the acyl product, rather than a direct stimulation of GOAT activity. That fluorophosphonates do not directly affect GOAT means that they are completely compatible with GOAT activity assays.

MAFP protects the acylated ghrelin peptide product through esterase inhibition

To confirm the mechanism for the MAFP-mediated enhancement of ghrelin acylation in the GOAT enzyme assay described above, the hydrolysis of the octanoylated ghrelin peptide and protection from hydrolysis by MAFP was examined. Octanoylated ghrelin peptide was generated through a preparative-scale hGOAT reaction in the presence of MAFP to enhance product formation, followed by product purification by reverse phase HPLC.[5] This octanoylated ghrelin peptide was then incubated with hGOAT-containing microsomal fraction treated with either MAFP or DMSO vehicle without the addition of octanoyl CoA. Thus, esterases present in the microsomal fraction would deacylate the peptide, but no GOAT-catalyzed acylation of the peptide would occur.

In the presence of untreated microsomal fraction, the octanoylated peptide was rapidly deacylated within 15 minutes and was almost completely deacylated after 1 hour. (Figure 2.3) In

the presence of MAFP-treated microsomal fraction, the octanoylated peptide was completely protected from hydrolysis and was stable over 5 hours of incubation. This protection of the acylated peptide is consistent with concomitant esterases within the microsomal fraction being responsible for the low levels of product formation seen in standard assays.

MAFP may protect octanoyl CoA from thioesterase-catalyzed hydrolysis

Many reported GOAT activity assays incorporate palmitoyl CoA, which is proposed to increase concentrations of the octanoylated product generated by competing for thioesterases within the microsomal fraction which might otherwise consume the octanoyl CoA substrate.[2, 4, 6-8] In the original development of our group's microsomal assay, a titration of octanoyl CoA revealed maximum octanoylation activity at a concentration of 500 μM octanoyl CoA.[5] The large excess of octanoyl CoA may have served the same purpose as the palmitoyl CoA used in other assays, acting as both substrate for the nonspecific thioesterases and for GOAT-catalyzed acylation of the ghrelin peptide.

We hypothesized that MAFP may inhibit thioesterases within the microsomal fraction, and that in the presence of MAFP, our assay would no longer require such a large excess of octanoyl CoA. A titration of octanoyl CoA in the presence of 1 μM MAFP revealed maximum octanoylation activity at 125 μM octanoyl CoA (Figure 2.4). A fit of the titration curve revealed a K_m of 36 ± 15 μM for octanoyl CoA, which is lower than the apparent K_m for octanoyl CoA in the absence of MAFP.[5]

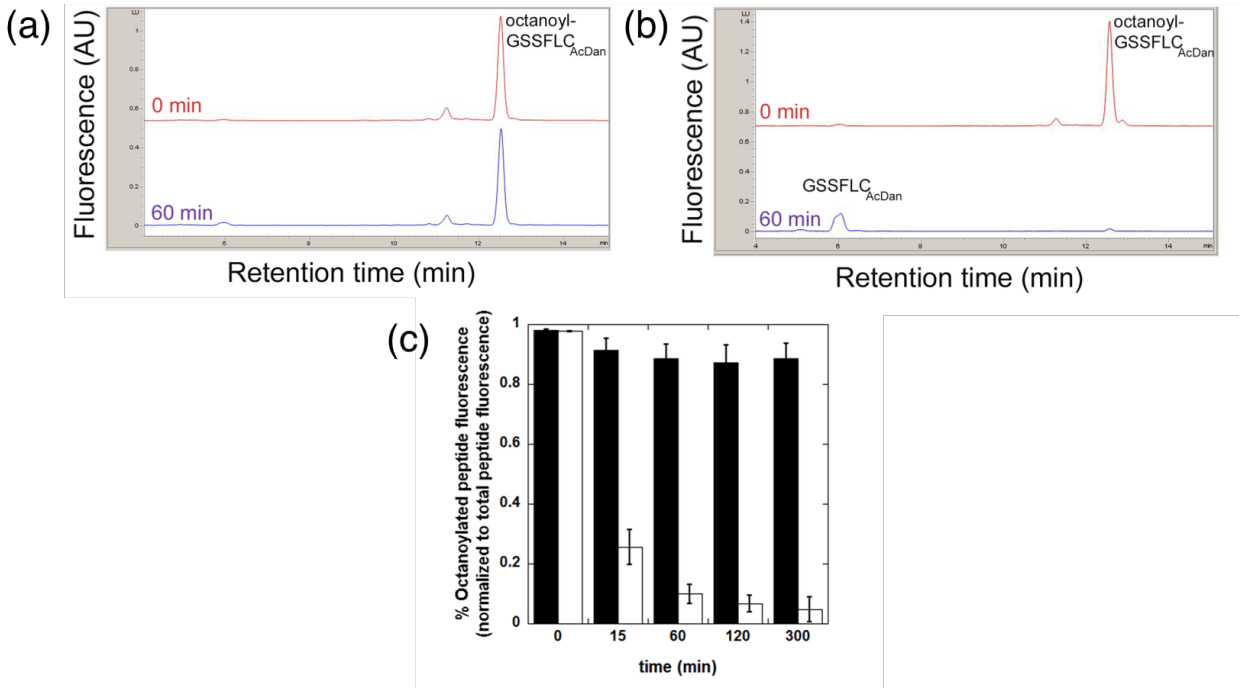

Figure 2.3 Octanoylated ghrelin mimetic peptide is protected from deacylation in the presence of MAFP. (a) HPLC chromatograms of octanoyl $GSSFLC_{AcDan}$ incubated with microsomal protein fraction pretreated with 5 μM MAFP. Top trace, 0 min incubation; bottom trace, 60 min incubation. (b) HPLC chromatograms of octanoyl $GSSFLC_{AcDan}$ incubated with microsomal protein fraction pretreated with DMSO (vehicle). Top trace, 0 min incubation; bottom trace, 60 min incubation. (c) Time dependence of octanoyl-$GSSFLC_{AcDan}$ deacylation. Filled bar, microsomal protein fraction pretreated with 5 μM MAFP; open bar, microsomal protein fraction pretreated with DMSO (vehicle). Octanoylated peptide fluorescence is reported as the percentage of total peptide fluorescence (octanoyl $GSSFLC_{AcDan}$ and unacylated $GSSFLC_{AcDan}$). Error bars represent the standard deviation from a minimum of 3 independent trials. Reprinted with permission from reference 1. © 2016, Oxford University Press.

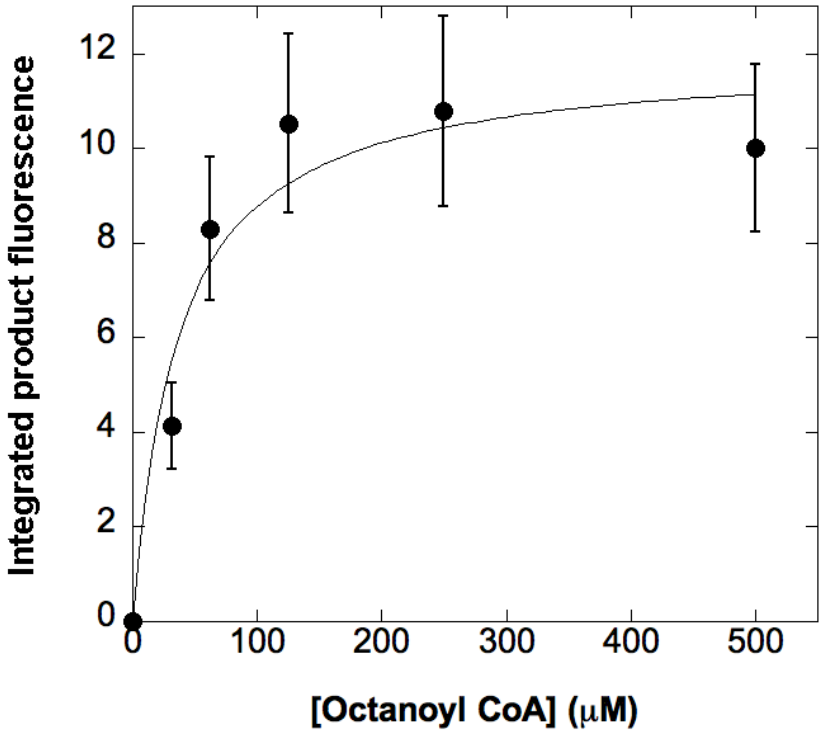

Figure 2.4 Dependence of ghrelin peptide octanoylation on concentration of octanoyl CoA in the presence of MAFP. Reactions were performed and analyzed as described in the Materials and methods section. Error bars represent the standard deviation from 3 independent trials.

MAFP treatment is compatible with ELISA measurements of acyl ghrelin concentrations

Most studies involving measurements of acyl ghrelin in biological samples rely on commercially available ELISA kits, which typically detect ghrelin with antibodies recognizing either the N- or C-terminus of the peptide.[6, 10, 12, 19] Standard curves for acyl ghrelin were generated in the presence and absence of MAFP to determine the compatibility of MAFP treatment with ELISA. Treatment with either 1 or 5 μM MAFP leads to a ~30% increase of the ELISA signal compared with buffer alone (Figure 2.5). This enhancement is consistent across the range of 100- to 2000-pg/ml acyl ghrelin recommended for the ELISA kit. Incorporation of MAFP into biological samples to stabilize acyl ghrelin from hydrolysis during sample workup is compatible with measurement of ghrelin by ELISA, as long as the standard curve is generated in the presence of MAFP as well.

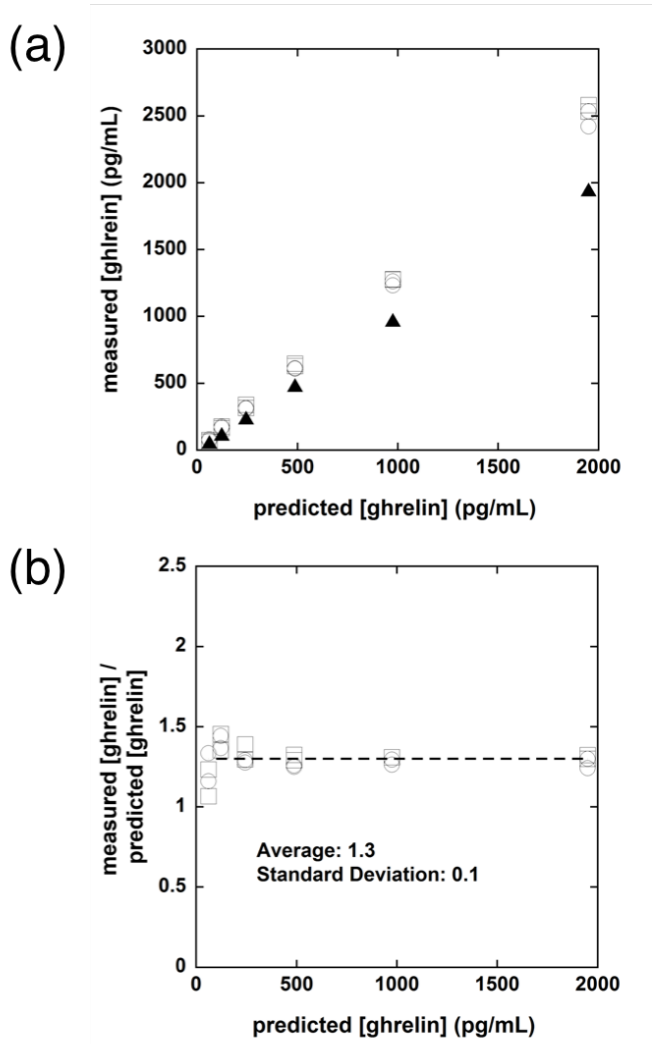

Figure 2.5 MAFP treatment is compatible with acyl ghrelin measurement by ELISA. (a) Standard curves generated by serial dilution of acyl ghrelin standards provided with a commercial acyl ghrelin ELISA kit (Millipore). Standard curves were measured in the presence of the DMSO vehicle alone (closed triangles) or after ELISA buffer treatment with either 1 μM MAFP (open squares) or 5 μM MAFP (open circles). Measured acyl ghrelin concentrations were calculated for each condition using the standard curve generated in the presence of DMSO alone. (b) Comparison of acyl ghrelin ELISA signal in the presence and absence of MAFP. For each predicted ghrelin concentration, the signal in the presence of MAFP (1 μM, open squares, 5 μM open circles) was normalized to the signal with the DMSO vehicle alone. Reprinted with permission from reference 1. © 2016, Oxford University Press.

MAFP treatment provides protection of acylated ghrelin in biological samples

The ability of MAFP to block acyl ghrelin hydrolysis in biological samples was assessed in collaboration with the Abizaid lab of Carleton University. The percentage of exogenous acylated ghrelin that is protected by MAFP treatment of HEK293FT cell lysate and of rat blood plasma was determined by ELISA. To ensure that MAFP does not affect acyl ghrelin levels detected in the absence of possible esterase activity, equal amounts of exogenous acylated ghrelin was added to mock samples composed of lysis or ELISA assay buffer in the presence or absence of MAFP. MAFP treatment does not markedly increase ghrelin levels compared with untreated controls in these samples (Figure 2.6, a and b, lanes 2 and 3). In untreated HEK293FT cell lysate, ghrelin is efficiently deacylated to background levels, whereas MAFP-treated lysate yielded complete protection of acyl ghrelin compared to buffer controls (Figure 2.6a, lanes 6 and 7). Similar results were seen in rat blood plasma, which was stored overnight at 4 °C to deplete endogenous ghrelin before treatment with MAFP or vehicle. Plasma treated with MAFP yielded nearly complete protection of exogenously added ghrelin, whereas ghrelin added to untreated plasma was nearly completely degraded (Figure 2.6b, lanes 6 and 7).

MAFP was also shown to be effective at protecting endogenous acyl ghrelin in rat blood samples. When added directly to the blood samples, MAFP treatment resulted in higher concentrations of acyl ghrelin detected compared to untreated samples, as well as samples treated with either AEBSF or HCl (Figure 2.6c), which are the current standard conditions for ghrelin stabilization.[9, 10, 12, 21]

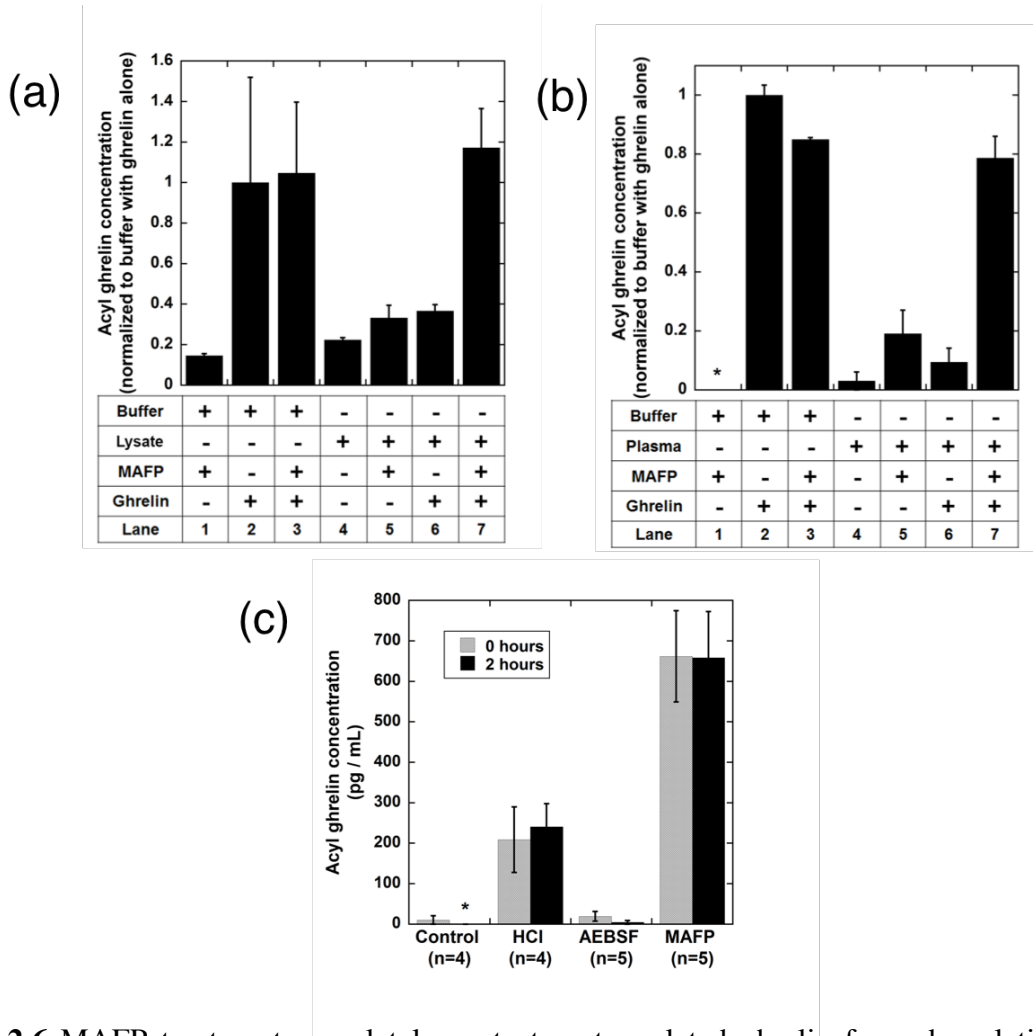

Figure 2.6 MAFP treatment completely protects octanoylated ghrelin from deacylation by mammalian cellular and blood esterases. (a) Protection of exogenous acyl ghrelin from esterase hydrolysis in human cell lysate. Acyl ghrelin concentrations are normalized to ELISA buffer treated with ghrelin alone. Lane 1, lysis buffer treated with MAFP; lane 2, lysis buffer treated with ghrelin; lane 3, lysis buffer treated with both MAFP and ghrelin; lane 4, untreated cell lysate; lane 5, cell lysate treated with MAFP; lane 6, cell lysate treated with ghrelin; lane 7, cell lysate treated with both MAFP and ghrelin. Error bars represent the standard deviation from at least 3 trials. (b) Protection of exogenous acyl ghrelin from esterase hydrolysis in rat blood plasma. Acyl ghrelin concentrations are normalized to ELISA buffer treated with ghrelin alone. Lane 1, ELISA buffer treated with MAFP; lane 2, ELISA buffer treated with ghrelin; lane 3, ELISA buffer treated with both MAFP and ghrelin; lane 4, untreated plasma; lane 5, plasma treated with MAFP; lane 6, plasma treated with ghrelin; lane 7, plasma treated with both MAFP and ghrelin. Error bars represent the standard error from a minimum of 4 biological replicates. *, acyl ghrelin not detectable by ELISA. (c) Protection of endogenous octanoylated ghrelin in rat blood samples. Blood samples were acidified by addition of HCl, treated with AEBSF or MAFP inhibitors, or left untreated before plasma separation by centrifugation. Acyl ghrelin concentrations were measured immediately after centrifugation (crosshatched bar) or after plasma incubation on ice for 2 hours (solid bar). Reprinted with permission from reference 1.

2.3 Discussion and conclusions

Ghrelin's susceptibility to deacylation by esterases has posed a major problem to the study of the hormone. During the early years following its discovery, concentrations of unacylated ghrelin in circulation were estimated to be much higher than those of acylated ghrelin.[22-24] As improved methods for stabilizing ghrelin from degradation in biological samples were reported, it became apparent that the ratio of unacylated to acylated ghrelin may actually favor the acylated form.[19] As unacylated ghrelin exerts biological effects,[25-30] accurate measurement of true circulating levels of each form of the hormone is of paramount importance. While acidification with HCl or treatment with protease inhibitors such as AEBSF offer some protection of the octanoyl modification by esterases within biological samples,[9, 10, 12, 21] the studies described above demonstrate that these treatments may not provide complete protection from hydrolysis. On the other hand, treatment with MAFP completely protects acylated ghrelin from deacylation in both HEK293FT cell lysate and rat blood plasma.

In vitro studies of GOAT activity also benefit from fluorophosphonate treatment. Concomitant esterases within the microsomal protein fraction enriched from cells expressing GOAT degrade the acyl product as it is formed, resulting in low levels of product formation and endpoints reached within 5 minutes to 1 hour, depending on the specific assay used. This limitation has hampered the study of GOAT activity. By inhibiting esterases present in the microsomal fraction used in these assays, the acylated product is protected from degradation, allowing for greater product accumulation and longer reaction times. Increasing the observed product formation and reaction times may enable the study of GOAT enzyme variants or ghrelin substrate variants with lower intrinsic reactivity. MAFP may also protect the acyl donor from hydrolysis catalyzed by thioesterases within the microsomal fraction, removing the need for large excess of octanoyl

CoA in our assay. Incorporation of MAFP in other microsomal GOAT assays may also render the addition of palmitoyl CoA unnecessary.[2, 4, 7, 8]

MAFP is a broad-spectrum esterase inhibitor, protecting ghrelin deacylation from both membrane-associated and soluble esterases. Its use should be applicable to all studies investigating the concentration of ghrelin *in vivo*, in cellular assays, and in *in vitro* GOAT activity assays. Comparison of endogenous acyl ghrelin concentrations in rat blood plasma treated with MAFP to those treated with either HCl or AEBSF indicate that the ratio of acylated to unacylated ghrelin may be much higher than previously estimated.[19] Given the physiological importance of both acylated and unacylated ghrelin, incorporation of effective esterase inhibitors such as MAFP or HDFP is necessary for the accurate determination of the true *in situ* ratio of the two forms of this hormone.

2.4 Materials and methods

General methods

Data plotting and curve fitting were performed with Kaleidagraph (Synergy Software). 4-(2-aminoethyl)benzenefulfonyl fluoride hydrochloride (AEBSF) was purchased from Cayman Chemical and solubilized in water immediately before use. Methoxy arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical as a stock in methyl acetate and diluted into dimethyl sulfoxide (DMSO) before use. Hexadecyl fluorophosphonate (HDFP) was generously provided by the Martin laboratory (Department of Chemistry, University of Michigan) and diluted into DMSO before use. Octanoyl CoA was solubilized to 5 mM in 10 mM Tris-HCl (pH 7.0), aliquoted into low-adhesion microcentrifuge tubes, and stored at -80 °C. Acrylodan (Anaspec) was solubilized in acetonitrile, with the concentration determined by absorbance at 393 nm on dilution into methanol (ε = 18,483 M^{-1} cm^{-1} per manufacturer's data sheet). The $GSSFLC_{NH2}$ peptide for fluorescent labeling with acrylodan was synthesized by Sigma-Genosys in the Pep-screen format, and solubilized in 1:1 acetonitrile:H_2O and stored at -80 °C. Peptide concentration was determined by absorbance at 412 nm following reaction of the cysteine thiol with 5,5'-dithiobis(2-nitrobenzoic acid) using ε = 14,150 M^{-1} cm^{-1}.[31]

Expression and enrichment of human GOAT (hGOAT)

A gene encoding hGOAT with a C-terminal triple tag (FLAG, HA, His_6) was cloned into the pFastBacDual vector (Invitrogen) using *Eco*RI and *Xba*I restriction sites, with the resulting vector used to produce the baculovirus expression system protocol (Invitrogen). Sf9 insect cells (5.0×10^8 cells in a 500 mL total culture volume) were infected with hGOAT baculovirus at a multiplicity of infection of 10 followed by protein expression for 40 hr at 28 °C shaking 150 rpm.

Cells were harvested by centrifugation (500 x *g*, 5 min), and freezing the pellet at -80 °C. The cell pellets were thawed on ice, resuspended in 25 mL lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.0), 1 mM sodium ethylenediamine tetraacetate (NaEDTA), 1 mM dithiothreitol (DTT), complete protease inhibitor (Roche), 10 μg/mL pepstatin A, and 100 μM bis(4-nitrophenyl)phosphate]. The resuspended cells were lysed with a Dounce homogenizer on ice, followed by removal of cell debris by centrifugation (3,000 x *g*, 4 °C, 10 min). The microsomal fraction was then isolated by ultracentrifugation of the supernatant (100,000 x *g*, 4 °C, 1 hr). The isolated microsomal fraction pellet was resuspended in 50 mM HEPES (pH 7.0) and stored in low-adhesion microcentrifuge tubes at -80 °C until use.

Peptide substrate fluorescent labeling

$GSSFLC_{NH2}$ (300 μM) and acrylodan (500 μM) were dissolved in 500 μL 1:1 50 mM HEPES buffer (pH 7.8):acetonitrile, followed by incubation at room temperature in the dark for 18 hr with shaking. Acrylodan-labeled peptide was purified by reverse phase HPLC (Zorbax Eclipse XDB column, 9.4 x 250 mm) using an isocratic mobile phase of water containing 0.05% trifluoroacetic acid (TFA) (65%) and acetonitrile (35%) flowing at 4.2 mL/min over 21 min. Labeled peptide eluted around 8 min, with the acrylodan labeled peptide detected by UV absorbance at 360 nm. Collected fractions containing labeled peptides were dried under vacuum at room temperature and resuspended in 1:1 acetonitrile:H_2O, and labeling was confirmed by MALDI-TOF mass spectrometry (Bruker Autoflex III, SUNY-ESF) using a matrix of saturated sinapinic acid in 0.1% TFA and 50 mM ammonium phosphate. The concentration of acrylodanylated peptide was determined by absorbance of acrylodan at 360 nm (ε = 13,300 M^{-1} cm^{-1}), and peptide aliquots were stored at -80 °C.

hGOAT activity assays and analysis

In each assay, microsomal fraction containing approximately 50 μg membrane protein (as determined by Bradford assay) was incubated with inhibitor at the indicated concentration or vehicle (DMSO or H_2O) for 30 min at room temperature in 50 mM HEPES (pH 7.0) before reaction initiation with 500 μM octanoyl CoA and 1.5 μM $GSSFLC_{AcDan}$. Assays were incubated at room temperature for the times indicated and stopped by addition of equal volume 20% acetic acid in isopropanol. Reaction solutions were clarified by protein precipitation with 16.7 μL of 20% trichloroacetic acid followed by centrifugation (1,000 x *g*, 1 min), with the resulting supernatant collected for analysis.

Assay samples were analyzed on an Agilent 1260 HPLC with a C18 reverse phase column (Zorbax Eclipse, 4.6 x 150 mm) using a gradient of 30% acetonitrile in 0.05% TFA to 63% acetonitrile in 0.05% TFA over 14 min, followed by 100% acetonitrile for 10 min. Fluorescent peptides were detected by fluorescence (λ_{ex} = 360 nm, λ_{em} = 485 nm), with the unacylated peptide eluting with a retention time of ~6 min and the octanoylated peptide product eluting with a retention time of ~12 min. Chromatogram analysis and peak integration was performed using Chemstation for LC (Agilent Technologies).

For octanoyl CoA titration experiments, reactions were performed and analyzed as described above with the following modifications. Microsomal fraction containing hGOAT was incubated with 1 μM MAFP in reaction buffer for 30 minutes before initiating the reactions with indicated concentrations of octanoyl CoA and 1.5 μM $GSSFLC_{AcDan}$. Reactions were incubated for 2 hours at room temperature before stopping. To determine K_m of octanoyl CoA in the presence

of MAFP, the plot of integrated product fluorescence versus [octanoyl CoA] was fit to equation 1 using Kaleidagraph (Synergy Software, Reading, PA, USA):

$$(1)\ \text{Integrated product fluorescence} = m1 \times \frac{[\text{octanoyl CoA}]}{K_m + [\text{octanoyl CoA}]}$$

MAFP protection of octanoylated GSSFLC$_{AcDan}$ in microsomal fraction

Octanoyl-GSSFLC$_{AcDan}$ was prepared using the standard hGOAT activity assay described above, in preparative scale (10 x 1 mL reactions) in the presence of 5 μM MAFP. The product was purified by reverse phase HPLC (Zorbax Eclipse XDB column, 9.4 x 250 mm) using an isocratic mobile phase of water containing 0.05% trifluoroacetic acid (TFA) (65%) and acetonitrile (35%) flowing at 4.2 mL/min over 21 min). The peptide substrate was monitored by absorbance of acrylodan at 360 nm, collected, and used as the substrate in protection assays.

For microsomal fraction esterase activity assays, approximately 50 μg microsomal protein from Sf9 cells transfected with hGOAT were incubated with either 5 μM MAFP or DMSO vehicle in 50 mM HEPES (pH 7.0) for 30 min at room temperature followed by addition of 1.5 μM octanoyl-GSSFLC$_{AcDan}$. Reactions were incubated for the indicated times, and were stopped with the addition of equal volume 20% acetic acid in isopropanol. Reaction solutions were clarified by protein precipitation with 16.7 μL of 20% trichloroacetic acid followed by centrifugation (1,000 x *g*, 1 min), with the resulting supernatant collected for analysis by reverse phase HPLC as described for hGOAT activity assays.

MAFP protection of exogenously added ghrelin in HEK293FT cell lysate

HEK293FT cells were cultured in DMEM medium (Corning) supplemented with 10% inactivated fetal bovine serum, 1% penicillin/streptomycin, and 0.01% octanoic acid. Cells were

routinely passaged 2-3 times per week. Cells were lysed at 3 x 10^5 cells/100 μL in lysis buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate, and Complete Protease Inhibitor Tab; Roche) with or without 5 μM MAFP as appropriate by vortexing for 3 seconds and freezing at -80 °C for a minimum of 16 hr. Immediately before use, lysates were thawed on ice, centrifuged at 20,000 x *g* for 20 min at 4 °C, and the supernatant was transferred to another 1.5 mL siliconized tube. Lysates were treated with 400 pg/mL human acyl ghrelin as appropriate and incubated on ice for 120 min before loading onto a human active ghrelin ELISA plate (EMD Millipore Corp). All samples were assayed in triplicate, with one standard deviation reported as error bars.

Research animals (Carleton University)

Male Wistar rats were bred and maintained in the Department of Neuroscience at Carleton University. Rats weighing between 350 and 400 g were used in this study. Before killing, all rats were housed in pairs in ventilated cages under standard laboratory conditions with a 12-hour light, 12-hour dark cycle with lights on at 7 AM. Animals had free access to standard rat chow (Purina) and tap water and were kept at constant humidity and temperature ranging from 21 °C to 23 °C. To obtain blood samples, animals were killed by CO_2 inhalation followed by rapid decapitation. All experimental manipulations were approved by the Carleton University Animal Care Committee and adhered to the standards of the Canadian Council for Animal Care.

MAFP protection of exogenously added ghrelin in rat blood plasma (Experiment performed by Trevor Rodrigues, Carleton University)

Male Wistar rats (n = 4) were used in this study. After killing, the blood sample from each animal was centrifuged, with plasma divided into 4 tubes and left untreated overnight at 4 °C. The next morning, the plasma samples from each animal were treated with the following additives: 1) vehicle alone; 2) vehicle and ghrelin (final concentration 400 pg/mL); 3) MAFP (final concentration, 5 μM); and 4) ghrelin (final concentration 500 pg/mL) followed by MAFP (final concentration, 5 μM). Ghrelin stability in assay buffer was also measured in the presence and absence of MAFP (final concentration, 5 μM); assay buffer with MAFP alone does not yield a detectable signal. After incubation on ice for 120 minutes, samples were assayed for acyl ghrelin concentrations using a commercially available acyl ghrelin ELISA (Millipore). All samples were assayed in duplicate. Variability between duplicates was less than 13%.

Inhibitor protection of endogenous ghrelin in rat blood samples (Experiment performed by Trevor Rodrigues, Carleton University)

Male Wistar rats (n = 10) were used in this study. Trunk blood from each rat was collected into 4 different borosilicate tubes containing EDTA. The first sample was treated with 1 N HCl (10 μL/mL of blood). The second sample was treated with AEBSF (10 μL/mL of blood from a 400 mM stock solution to yield a final concentration of 4 mM [0.9 mg/mL]); the third sample was treated with MAFP [10 μL/mL of blood from a 500 μM stock solution to yield a final concentration of 5 μM (1.85 μg/mL)]. The last sample (control) was not treated. Samples were treated with described ghrelin deacylation inhibition conditions immediately upon blood collection. The samples from 5 rats were centrifuged for 5 min at 3,000 rpm and incubated on ice for 2 hr before

being frozen and stored overnight at -20 °C. The samples from additional 5 rats were centrifuged after treatment and stored immediately at -20 °C. The next morning, samples were thawed and processed for acyl ghrelin quantification using a commercially available acyl ghrelin ELISA (Millipore). All samples were assayed in duplicate. Variability between duplicates was less than 10%.

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Chapter 3: Identification and development of small molecule hGOAT inhibitors

Portions of this chapter have been previously published and reprinted with permission from reference 1: McGovern-Gooch, K. R., Mahajani, N. S., Garagozzo, A., Schramm, A. J., Hannah, L. G., Sieburg, M. A., Chisholm, J. D., and Hougland, J. L. (2017), Synthetic triterpenoid inhibition of human ghrelin *O*-acyltransferase: The involvement of a functionally required cysteine provides mechanistic insight into ghrelin acylation. *Biochemistry*, 56:919-931. Copyright 2017, American Chemical Society. N. Mahajani synthesized compounds **9-14**, **16** and **17**. A. Garagozzo, A. J. Schramm, and L. G. Hannah contributed to library screening. M. A. Sieburg performed cell-based inhibition and cytotoxicity assays.

3.1 Introduction

Ghrelin's role in diseases such as diabetes, obesity, and Prader-Willi Syndrome highlights the need to explore treatment avenues to modulate this pathway. There are several potential targets within the ghrelin signaling pathway: antagonists of GHSR1a prevent ghrelin-induced growth hormone secretion; anti-ghrelin antibodies and Spiegelmers bind and sequester ghrelin in circulation; and inhibition of GOAT prevents the acylation of ghrelin, preventing its ability to bind and activate GHSR1a. In addition, mimics of unacylated ghrelin have been shown to have biological effects, though their target remains unknown.

Both antagonists and agonists of GHSR1a have been reported as potential therapeutics for conditions related to growth hormone signaling, with varying degrees of success in clinical and preclinical trials.[2-4] Agonists, which bind to GHSR1a and stimulate the release of growth hormone, have been developed for conditions such as cachexia and anorexia,[5-7] age-related declines in muscle mass,[8, 9] and gastroparesis related to various conditions.[10, 11] Antagonists or inverse agonists of GHSR1a prevent its release of growth hormone, and have potential to treat conditions such as obesity,[12-15] diabetes,[13, 14] and addictive behaviors.[16-20]

Antagonism of ghrelin signaling has also been achieved through a vaccination approach. Rats producing anti-ghrelin antibodies exhibited decreased weight gain as a result of selective binding of acylated ghrelin in plasma.[21] RNA-based Spiegelmers are also able bind to circulating acyl ghrelin, preventing growth hormone release in rats,[22] inhibiting ghrelin-induced food foraging and hoarding behavior in Siberian hamsters,[23] and promoting weight loss and reducing ghrelin-induced food intake in diet-induced obese mice.[24] Administration of an anti-ghrelin Spiegelmer to neonatal mice was used to show that altered ghrelin signaling in the early stages of development leads to widespread disruption of the neural network in the arcuate nucleus of the hypothalamus,

providing a possible explanation for the lack of phenotypic changes in ghrelin knockout models.[25] Therapeutic applications of anti-ghrelin vaccines or Spiegelmers beyond these studies remain unexplored.

Inspired by the antagonistic effects of unacylated ghrelin on ghrelin-induced hyperglycemia and insulin resistance, several research groups have explored unacylated ghrelin analogs as therapeutics for diabetes. AZP531, a cyclic peptide consisting of residues 6–13 of ghrelin, prevented insulin resistance and attenuated adiposity induced by a high-fat diet in mice.[26] It exhibited similar effects in overweight humans, improving insulin sensitivity and increasing weight loss without affecting food intake.[27] Another unacylated ghrelin analog featuring the first ten residues of ghrelin with an S3A mutation followed by a Tat sequence for cell permeability, CF801, was shown to inhibit acyl ghrelin secretion from SG-1 (stomach) cells.[28] Mice treated with CF801 gained less weight, while consuming the same number of calories, when fed a high-fat diet compared to vehicle-treated mice.[28]

Lastly, inhibition of GOAT is a potential avenue for modulating ghrelin signaling *in vivo*. Acylation of serine 3 is absolutely required for ghrelin to bind and activate GHSR1a,[29] and bioinformatics analysis reveals that ghrelin is GOAT's only substrate.[30] Thus, inhibiting GOAT to reduce acyl ghrelin levels would be predicted to have limited off-target effects. The few GOAT inhibitors reported in the scientific literature as of the beginning of this project in 2012 are limited in their therapeutic potential. Many are peptide-based product or substrate mimetics,[31, 32] which may be unable to cross the cell membrane and may even bind to and activate GHSR1a.[33] GO-CoA-Tat, a bisubstrate analog consisting of ghrelin's N-terminal sequence stably linked to an octanoyl CoA group and a Tat peptide sequence to enable cell permeability, is currently the only inhibitor reported to be effective in cell and animal studies.[34] The ability of GO-CoA-Tat to lower acyl

ghrelin *in vivo* and decrease food intake, weight gain, and insulin resistance[34-36] establishes inhibition of GOAT as a viable therapeutic route to modulating ghrelin signaling.

Small molecule inhibitors of GOAT are especially attractive, as they are more likely than large peptidomimetics like GO-CoA-Tat to be orally available.[37] The only small molecule GOAT inhibitors reported in the scientific literature have only demonstrated efficacy in a microsomal assay.[38] The discovery of new small molecule GOAT inhibitors will advance the field toward the development of orally available therapeutics targeting ghrelin signaling, as well as providing a tool for studying the effects of GOAT inhibition *in vivo*.

As the active site structure of GOAT is not known, we sought to identify new small molecule inhibitors of hGOAT through screening a library of small molecules. After discovery of lead compounds, structure-activity analysis of the best candidate molecule revealed this inhibitor acted by reversibly alkylating a nucleophilic cysteine residue within the enzyme. This was the first demonstration that hGOAT may contain a functionally essential cysteine. However, the mouse isoform of GOAT is not susceptible to these or other cysteine-modifying inhibitors, indicating an unexpected mechanistic distinction between the two closely related enzymes.

3.2 Results

Screening of Diversity Set IV library reveals lead compounds

To identify novel small molecule inhibitors of hGOAT, we used our HPLC-based fluorescence assay to screen compounds from the Diversity Set IV small molecule library from the NIH's Developmental Therapeutics Program (DTP).[39, 40] This library contains 1,596 compounds representing diverse chemical scaffolds from the DTP repository. Compounds were initially screened at 10 and 100 μM, with compounds exhibiting dose-dependent inhibition and ≤ 50% activity relative to the vehicle control selected for a secondary screen under the same conditions (Figure 3.1). To date, 478 compounds from the library have been screened, and 14 have satisfied these criteria.

The most promising candidate molecule from this initial screen was identified as 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im), a synthetic oleanate triterpenoid derived from oleanolic acid.[41] A closely structurally related compound, CDDO-Me, also inhibited hGOAT activity (Figure 3.2). Therefore, we chose to pursue structure-activity analysis of this scaffold.

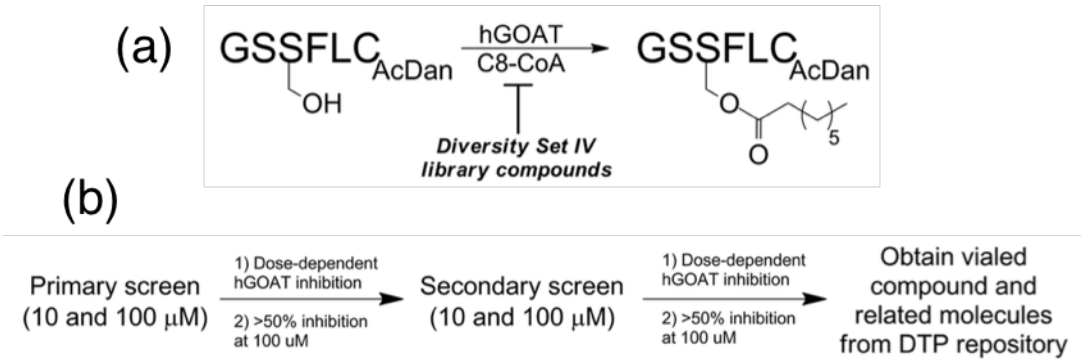

Figure 3.1 Screening format for identification of novel small molecule inhibitors of hGOAT. (a) Fluorescence-based hGOAT activity assay used for compound screening. (b) Protocol for the screening assay to identify hGOAT inhibitors. Reprinted with permission from reference 1. © 2017, American Chemical Society.

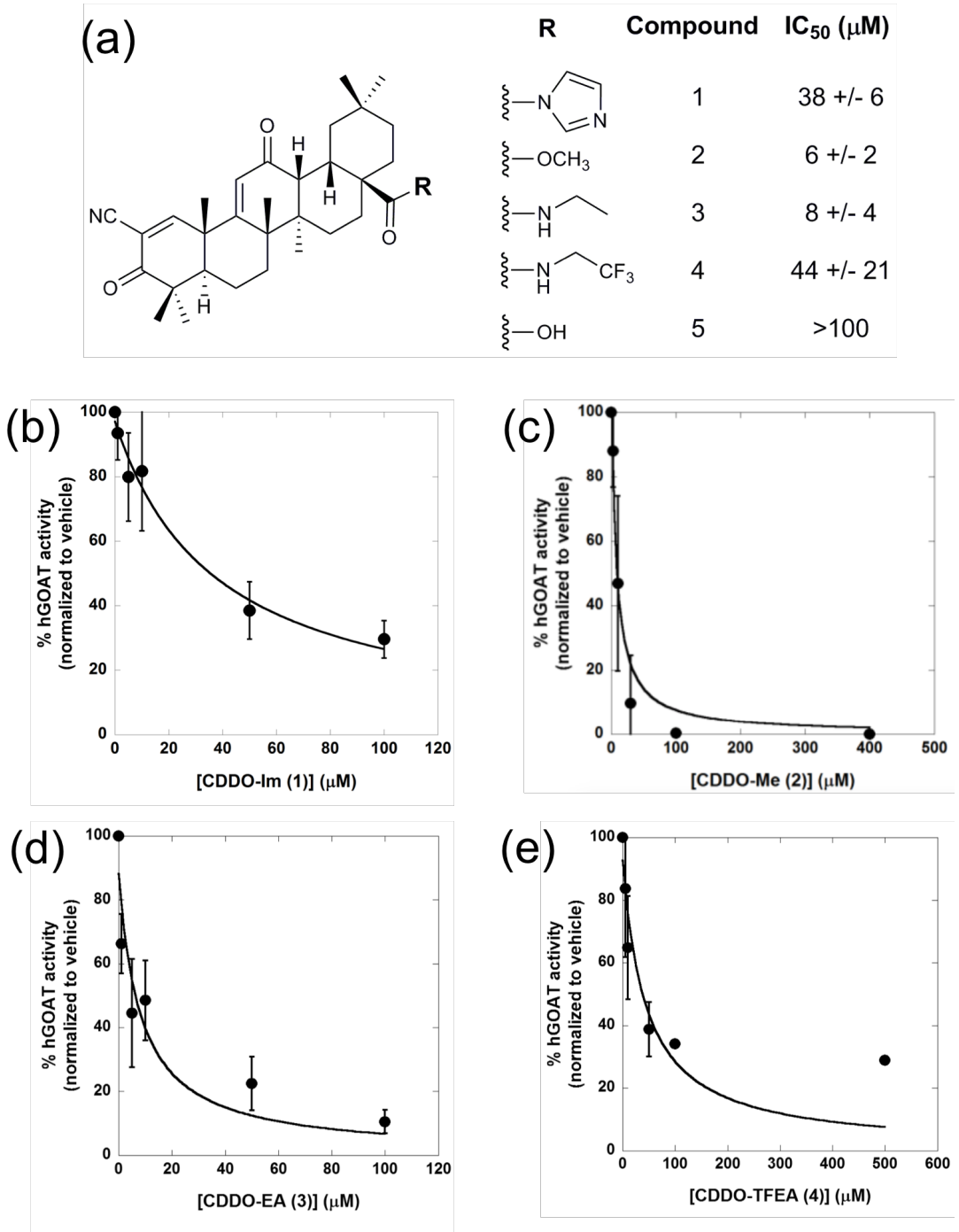

Figure 3.2 Multiple CDDO derivatives effectively inhibit hGOAT. (a) Structures and measured IC_{50} values for CDDO derivatives with substitutions at the carboxyl group at position 28: R = imidazole (CDDO-Im), R = methyl ester (CDDO-Me), R = ethylamide (CDDO-EA), R = trifluoroethylamide (CDDO-TFEA), and R = carboxylic acid (CDDO). (b-e) Inhibition of hGOAT octanoylation activity by (b) CDDO-Im, (c) CDDO-Me, (d), CDDO-EA, and (e) CDDO-TFEA. Reactions were performed and analyzed to determine percent activity. Error bars reflect the standard deviation from a minimum of three independent measurements. Reprinted with permission from reference 1. © 2017, American Chemical Society.

Structure-activity analysis of hGOAT inhibition by CDDO family compounds

In addition to CDDO-Im and CDDO-Me, this class of synthetic triterpenoids also contains CDDO, CDDO-EA, and CDDO-TFEA. (Figure 3.2) These orally available compounds have demonstrated antiangiogenic and antitumor activities by modulating the Nrf2- and NF-κB-dependent cellular signaling pathways.[41-44] We received these three additional compounds as a gift from the Gribble lab at Dartmouth College and tested each for activity against hGOAT. All but the parent molecule, CDDO, inhibited ghrelin acylation (Figure 3.2). The lack of activity by CDDO, which bears a carboxylate group, may reflect the inability of hGOAT to accept negative charge within its active site.[30, 31]

To further explore the structural parameters determining the CDDO-based inhibitors' activity against hGOAT, we evaluated the activity of several structural analogs of these compounds (Figure 3.3). Natural triterpenoids ursolic acid (**6**), oleanolic acid (**7**), and taraxerol (**8**) do not inhibit hGOAT activity at concentrations ≤ 100 μM. All three of these compounds share the triterpenoid scaffold of the CDDO class of inhibitors, suggesting that this scaffold is not sufficient for inhibitor potency. Importantly, taraxerol (**8**) does not feature a carboxylate group, as ursolic acid (**6**) and oleanolic acid (**7**) do, which further supports that the triterpenoid scaffold is not sufficient for activity.

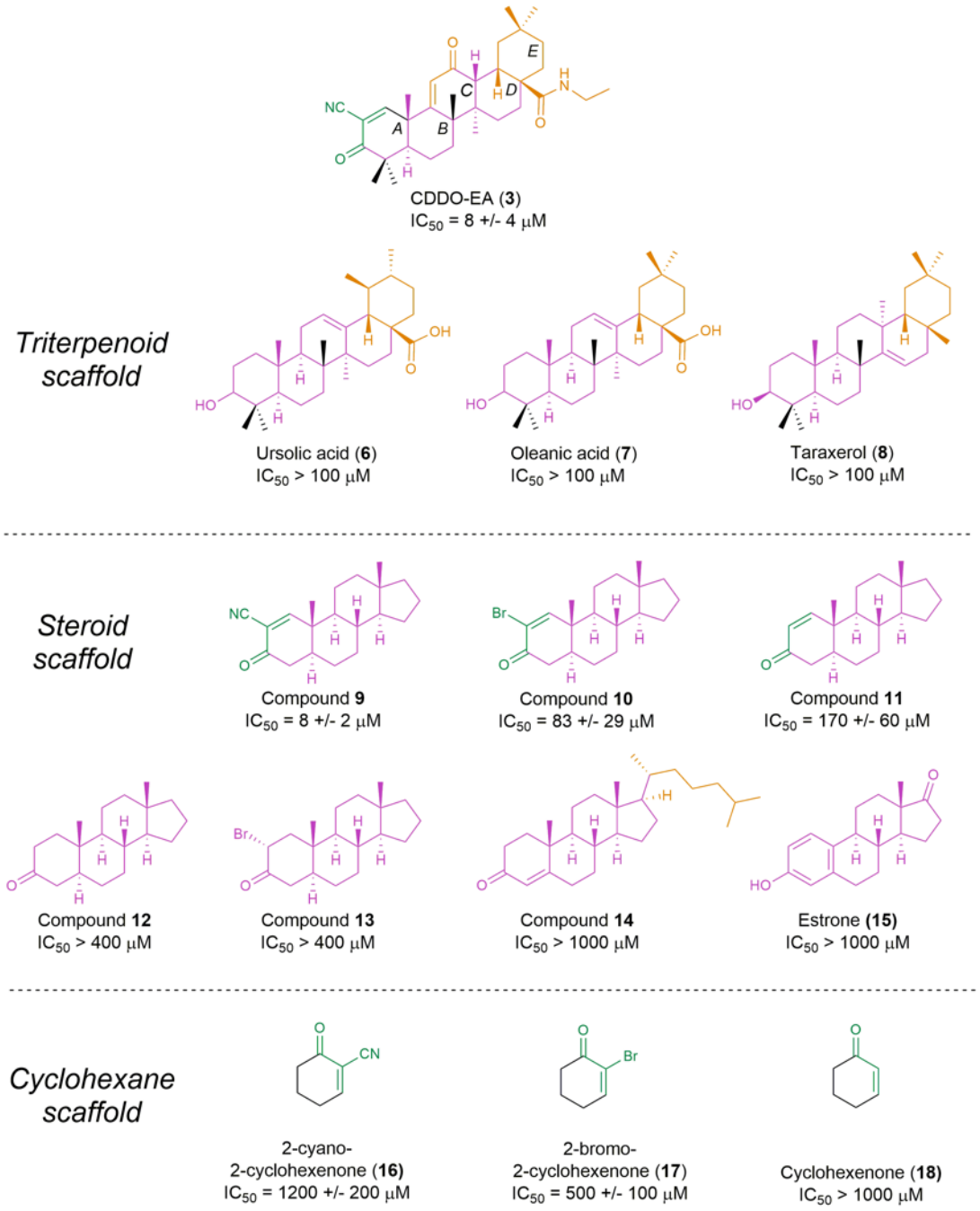

Figure 3.3 Structure activity analysis reveals multiple pharmacophores contribute to inhibition of hGOAT by synthetic triterpenoids. Compounds organized by overall hydrocarbon skeleton family (triterpenoid, steroid, or cyclohexane parent structure) and colored to reflect potential pharmacophores (α,β-unsaturated ketone, green; steroid scaffold, purple; CDDO derivative functional groups in rings C-E, orange). Measured IC_{50} values are provided for each compound, with lower limits established based on compound solubility and lack of inhibition observed at the highest experimentally accessible concentration. Reprinted with permission from reference 1. © 2017, American Chemical Society.

The α-cyanoenone moiety on the A ring of the CDDO molecules has been shown to modify nucleophilic cysteine residues within receptors to exert their antitumor activities.[45] Therefore, we hypothesized this functional group may be required for hGOAT inhibition. To test this hypothesis, a series of minimally functionalized steroid derivatives featuring an α,β-unsaturated ketone in the position analogous to that in the CDDO derivatives was synthesized by Nivedita Mahajani of the Chisholm lab. Compound **9**, which features the strongly electron-withdrawing α-cyano group present in the CDDO derivatives, inhibits hGOAT with an IC_{50} of 8 ± 2 μM, nearly identical to that of CDDO-Me and CDDO-EA (Figures 3.3 and 3.4a). The similar activity of compound **9** and CDDO-Me and -EA indicates that the complete triterpenoid scaffold and other functional groups within the CDDO molecule are not necessary for inhibitor potency. Compounds **10** and **11** bear a less strongly electron-withdrawing bromine and a non-electron-withdrawing hydrogen, respectively, at this position. Both inhibit hGOAT, though less strongly than compound **9**, with inhibitor potency scaling with level of electrophilic activation of the enone to nucleophilic addition (Figures 3.3 and 3.4b-c). Removal of the unsaturation in either the isolated ketone (compound **12**) or the α-bromo ketone (compound **13**) result in complete loss of inhibitory activity at concentrations ≤ 400 μM. Moving the unsaturation to the other side of the ketone (compound **14**) similarly abrogates activity, indicating that hGOAT inhibition exhibits regioselectivity toward the enone. The lack of inhibition by estrone (**15**) demonstrates that the planar conformation of the A ring is not sufficient for inhibition (Figure 3.3).

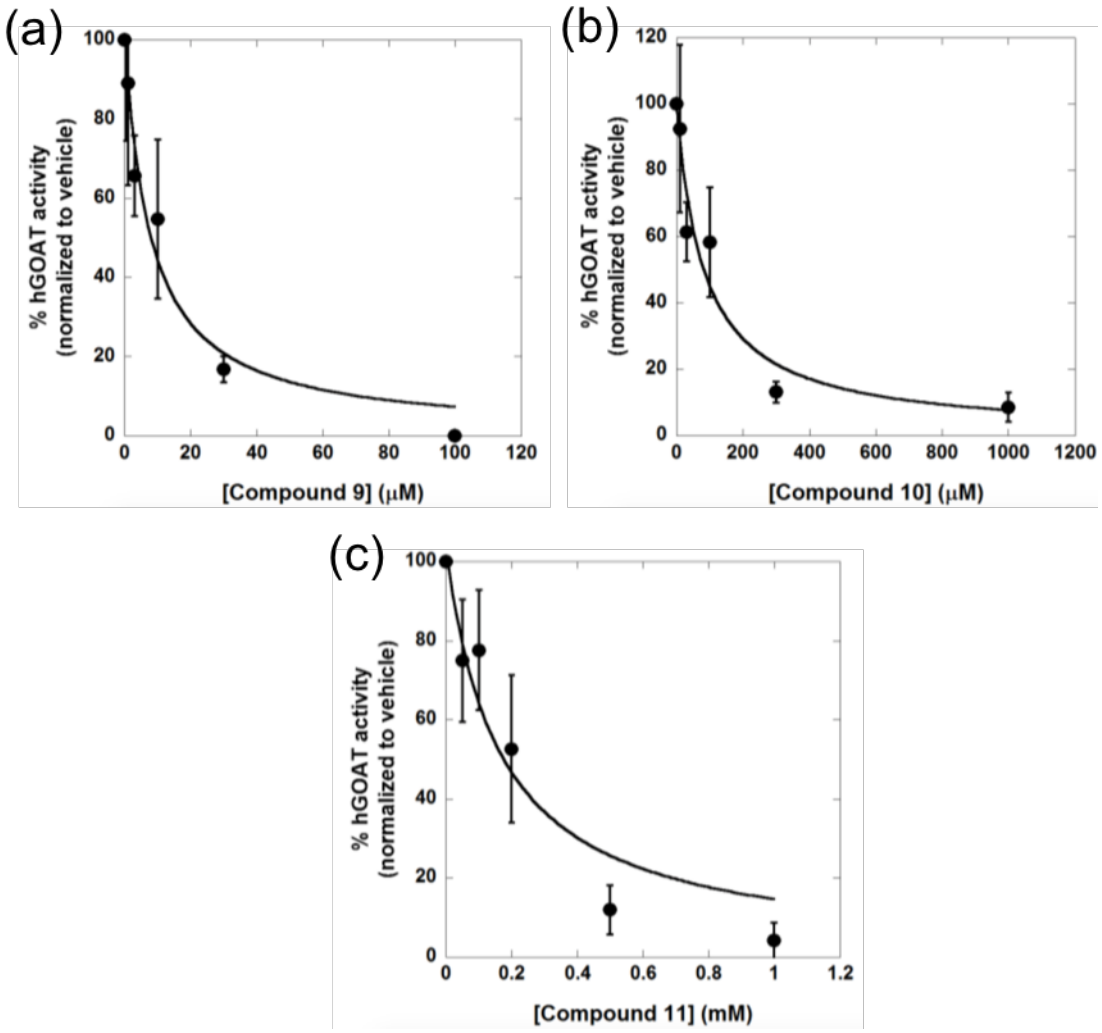

Figure 3.4 Inhibition of hGOAT by steroid derivatives. (a) Inhibition of hGOAT octanoylation activity by compound **9**. (b) Inhibition of hGOAT octanoylation activity by compound **10**. (c) Inhibition of hGOAT octanoylation activity by compound **11**. Reactions were performed and analyzed to determine percent activity as described in the Materials and methods section. Error bars reflect the standard deviation from a minimum of three independent measurements. Reprinted with permission from reference 1. © 2017, American Chemical Society.

We next determined the contribution of the steroid scaffold to inhibitor potency of compounds **9-11** by measuring inhibition of cyclohexenone derivatives **16-18**, which feature the same series of activation (Figures 3.3 and 3.5). Both α-cyanocyclohexenone (**16**) and α-bromocyclohexenone (**17**) inhibit hGOAT less potently than their steroid analogs, and the nonactivated cyclohexenone (**18**) does not inhibit hGOAT at concentrations ≤ 1 mM, indicating that the steroid scaffold contributes significantly to inhibitor potency. This contribution likely arises from a combination of enhanced binding of the inhibitor to the enzyme and decreased reactivity of the inhibitor with other proteins within the microsomal fraction.

CDDO derivatives inhibit hGOAT through a reversible, covalent modification of a functionally essential cysteine residue

The necessity for an activated enone within the CDDO-based hGOAT inhibitors suggests a mechanism involving the alkylation of a nucleophilic cysteine residue involved in hGOAT catalysis. We determined that hGOAT is susceptible to inactivation by *N*-ethylmaleimide (NEM), a common thiol-modifying reagent (Figure 3.6), supporting the involvement of a cysteine residue in ghrelin acylation.

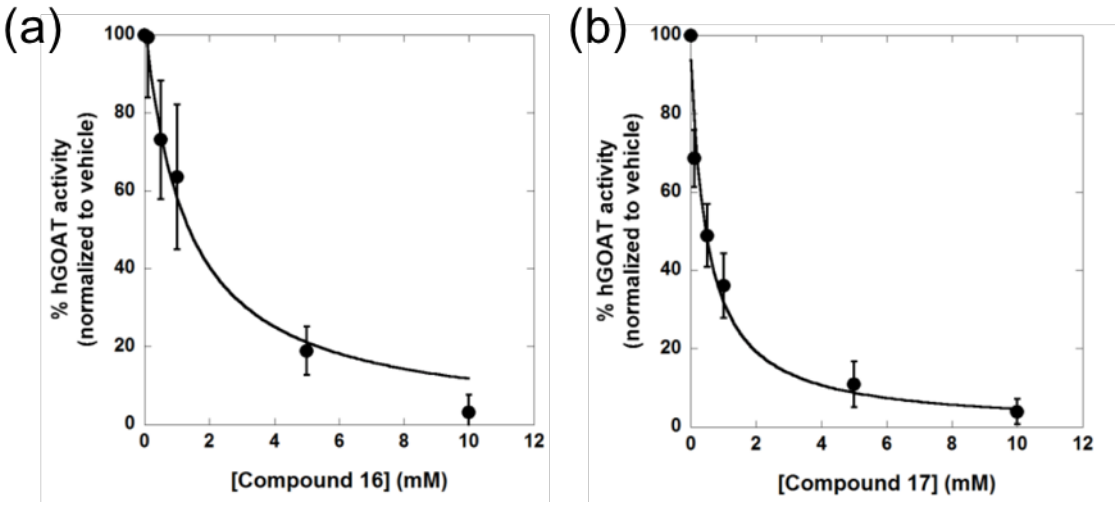

Figure 3.5 Inhibition of hGOAT activity by cyclohexenone derivatives. (a) Inhibition of hGOAT octanoylation activity by compound **16**. (b) Inhibition of hGOAT octanoylation activity by compound **17**. Reactions were performed and analyzed to determine percent activity as described in the Materials and methods section. Error bars reflect the standard deviation from a minimum of three independent measurements. Reprinted with permission from reference 1. © 2017, American Chemical Society.

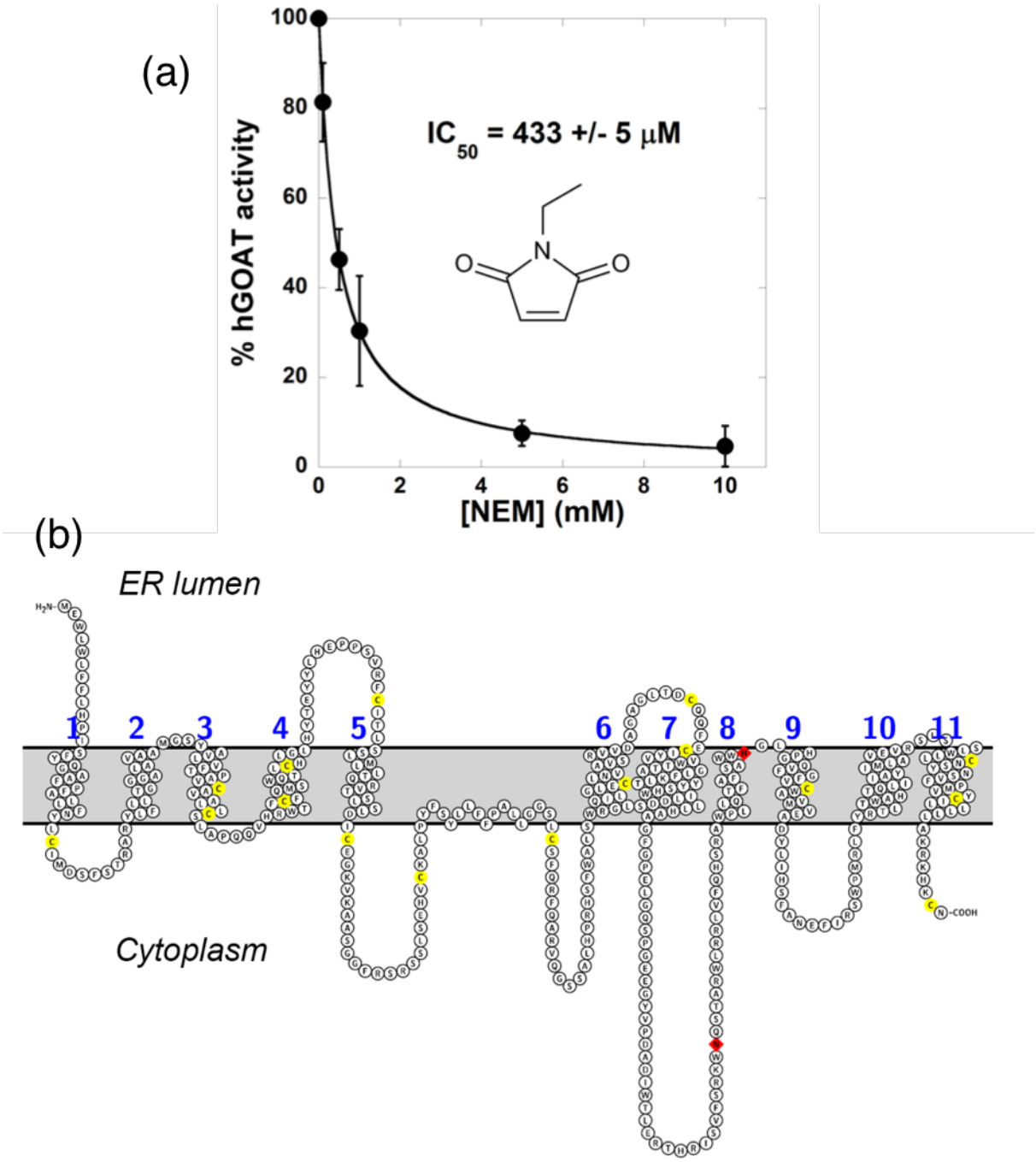

Figure 3.6 hGOAT inhibition profile supports the involvement of a catalytically essential cysteine residue. (a) hGOAT octanoylation activity is inhibited by *N*-ethylmaleimide (NEM, shown in inset). Error bars reflect the standard deviation of three independent determinations. (b) Topological model of hGOAT, with cysteine residues colored yellow and conserved functionally essential residues N307 and H338 colored red. This model was constructed by comparison to the experimentally developed topology model for mouse GOAT using the Protter online server.[46, 47] Reprinted with permission from reference 1. © 2017, American Chemical Society.

CDDO has been shown to react with nucleophilic thiols selectively at the α-cyanoenone of ring A through a covalent reversible Michael addition, with the retro-Michael addition facilitated by the increased acidity of the α proton geminal to the cyano group.[45, 48] Covalent modification by these inhibitors is supported by the time-dependent inhibition of hGOAT (Figure 3.7a). In this mechanism, a noncovalent enzyme-inhibitor complex is rapidly formed, followed by a slower covalent alkylation of the cysteine thiol by the α-cyanoenone. At short preincubation times, these inhibitors are less effective as evidenced by a higher IC_{50} value. The reversibility of inhibition by CDDO-EA and compound **9** was demonstrated through a pulse-dilution experiment, in which hGOAT was preincubated with the inhibitor at 3 times its IC_{50} concentration followed by a 10-fold dilution into either reaction buffer or buffer containing the same inhibitor concentration (Figure 3.7b). NEM exhibits irreversible hGOAT inhibition, with no increase in hGOAT activity following dilution of the inhibitor, consistent with irreversible cysteine alkylation. [Dap^3]octanoyl-ghrelin (1-5)-NH_2, a non-covalent product mimetic inhibitor,[31] exhibits expected reversible inhibition with the observed hGOAT activity increasing after inhibitor dilution. Both CDDO-EA and compound **9** exhibited reversible inhibition, supporting that these compounds inhibit hGOAT through the covalent Michael addition of nucleophilic cysteine residue.

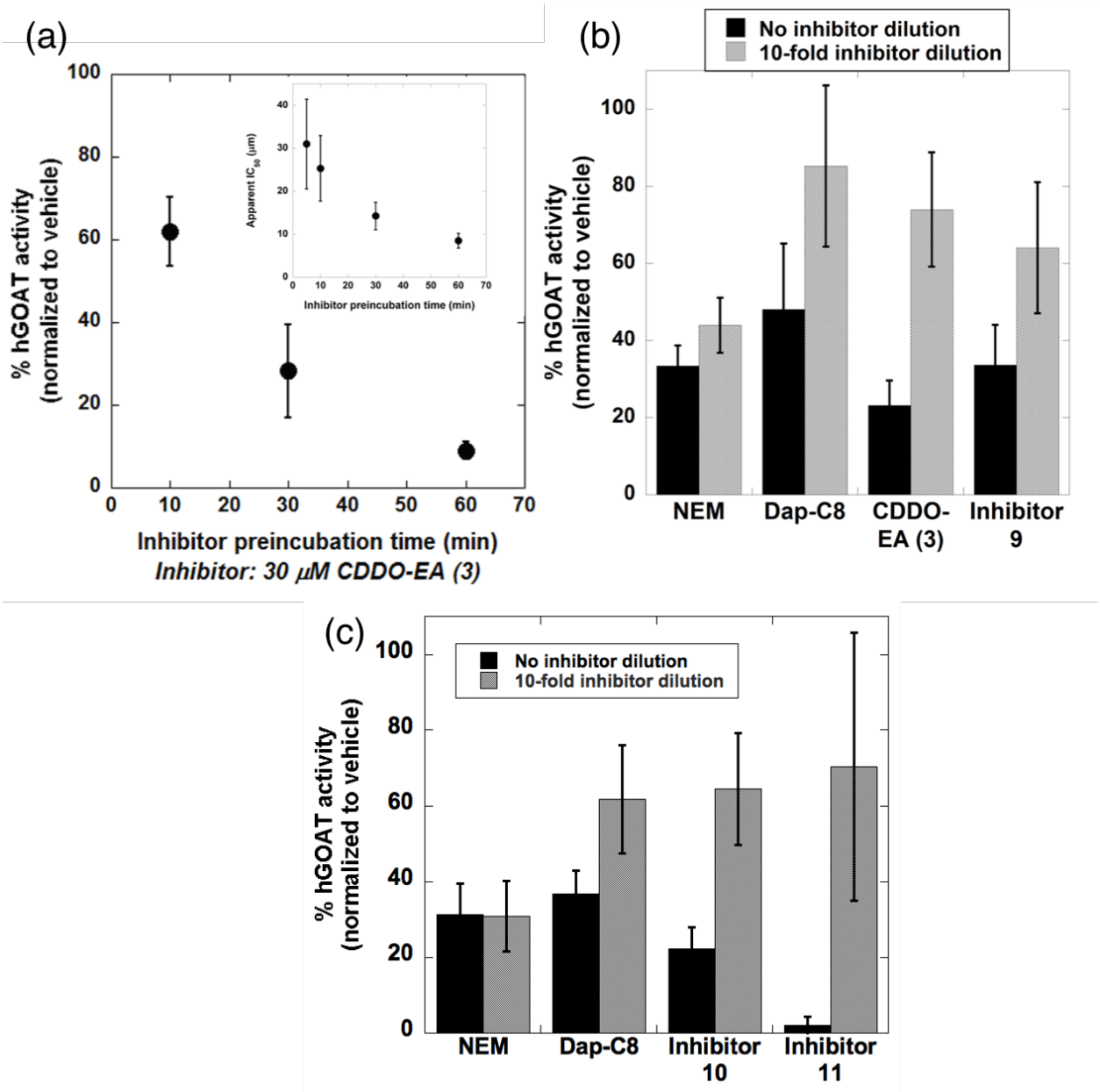

Figure 3.7 hGOAT inhibition profile supports a covalent reversible mechanism of inhibition. (a) Time dependence of hGOAT inhibition by CDDO-EA. hGOAT activity was measured as a function of preincubation time in the presence of 30 μM CDDO-EA. The inset shows IC_{50} values for CDDO-EA inhibition of hGOAT activity as a function of inhibitor preincubation time. (b) Inhibitor dilution assays reveal irreversible hGOAT inhibition by NEM and reversible inhibition by CDDO-EA and α-cyanoenone steroid **9**. (c) Inhibitor dilution assays reveal reversible hGOAT inhibition by steroids **10** and **11**. Dap-C8 denotes [Dap^3]octanoyl-ghrelin(1-5)-NH_2 product-mimetic hGOAT inhibitor used as a control for reversible inhibition. Error bars reflect the standard deviation from a minimum of three determinations. (a) and (b) reprinted with permission from reference 1.

Steroid-based inhibitors **10** and **11** also exhibited reversible inhibition, with hGOAT activity increasing after inhibitor dilution (Figure 3.7c). Given the lack of an electron-withdrawing group in compound **11** and the decreased acidities of the α proton in the resulting conjugate with hGOAT relative to compound **9**, inhibition by compound **11** was expected to be irreversible. While compound **10** features a moderately electron-withdrawing bromine at the α position, its reversible behavior was also somewhat surprising.

CDDO-Me and CDDO-EA decrease acyl ghrelin concentrations in HEK293FT cells

There are currently no small molecule GOAT inhibitors reported to be effective in cell or animal models, with only GO-CoA-Tat demonstrating the ability to lower acyl ghrelin levels in cell and animal models.[34] CDDO derivatives have been extensively studied in multiple biological systems, and their oral availability is well-established.[41-43] The most potent CDDO derivatives against hGOAT in our enzyme activity assay, CDDO-Me and CDDO-EA, were chosen to determine their ability to inhibit GOAT-catalyzed acylation of ghrelin in a cellular assay.

HEK293FT cells stably transfected to express preproghrelin and the mouse isoform of GOAT (mGOAT) and were treated with CDDO, CDDO-Me, or CDDO-EA.[34] Cellular acyl ghrelin levels were determined by ELISA. CDDO, which was not active against hGOAT in the enzymatic assay, did not significantly lower acyl ghrelin levels at concentrations below 1 μM (Figure 3.8a). Both CDDO-Me and CDDO-EA reduced acyl ghrelin levels with cellular IC_{50} values of 35 ± 10 nM and 320 ± 150 nM, respectively. These concentrations for effective reduction of acyl ghrelin are far below the onset of cytotoxicity. Apparent LD_{50} values for CDDO-Me and CDDO-EA were both around 10 μM in the HEK293FT cells used (Figure 3.8b).

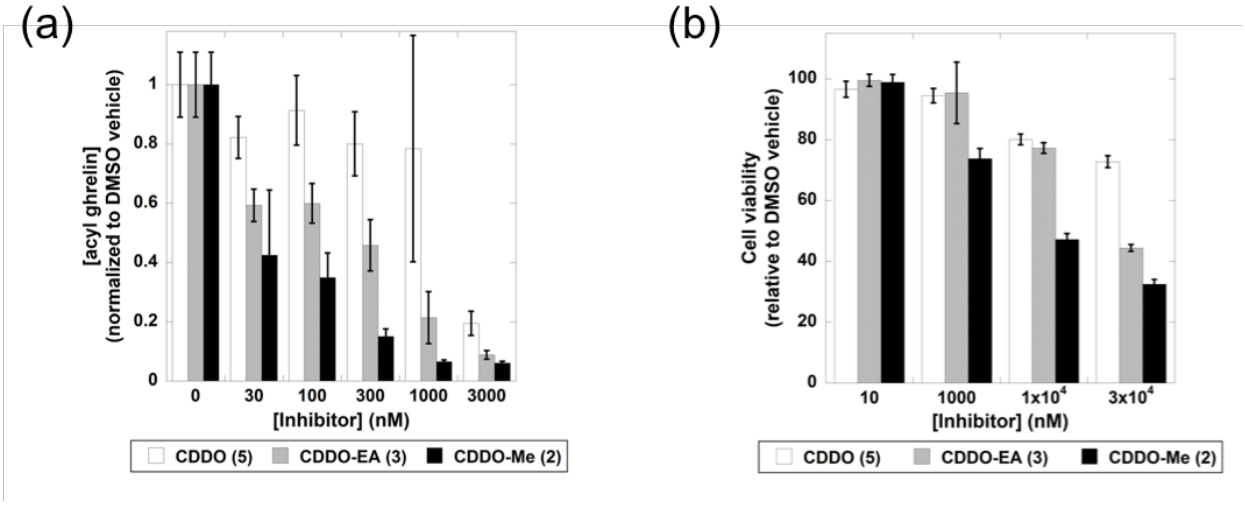

Figure 3.8 Acyl ghrelin levels are decreased by treatment with CDDO-EA and CDDO-Me in a cellular assay. (a) Dose-dependent reduction of acyl ghrelin concentrations in mGOAT/preproghrelin transfected HEK293FT cells following 24 hours incubation, normalized to the DMSO vehicle. CDDO, white bars; CDDO-EA, gray bars; and CDDO-Me, black bars. Error bars reflect the standard deviation from three independent measurements. (b) Cell viability for mGOAT/preproghrelin transfected HEK293FT cells following 24 hours incubation, normalized to the DMSO vehicle. CDDO, white bars; CDDO-EA, gray bars; and CDDO-Me, black bars. Error bars reflect the standard deviation from three independent measurements.

Mouse GOAT is not susceptible to cysteine-modifying inhibitors

The effective concentrations of both CDDO-Me and CDDO-EA for lowering acyl ghrelin levels in the cellular assay were substantially lower than those determined in the hGOAT activity assay. This discrepancy could arise from increased susceptibility of mGOAT used in the cellular assays to inhibition by these compounds compared to hGOAT, which was used for all enzyme activity assays.

Unexpectedly, we found that CDDO-EA inhibits mGOAT less potently than it inhibits hGOAT, with an IC_{50} value of 60 ± 8 μM for the mouse isoform (Figure 3.9). To explore the cause of this loss of potency, we determined whether inhibition of mGOAT exhibits the same chemoselectivity for the electrophilic enone demonstrated for hGOAT. Both enzymes were treated with α-cyanoenone **9** or ketone **12**. These steroid derivatives differ only in the α-nitrile and α,β-unsaturation of ring A (Figure 3.3), so any difference in inhibition activity against GOAT can be directly attributed to the enzyme's chemoselectivity for the α-cyanoenone. Compound **9** potently inhibits hGOAT, while ketone **12** is inactive at concentrations ≤ 100 μM, indicating the electrophilic enone is providing an enhancement in potency. In contrast, the presence of the cyanoenone does not provide an enhancement of potency against mGOAT (Figure 3.10a). In addition, mGOAT is resistant to inhibition by NEM in contrast to hGOAT (Figure 3.10b).

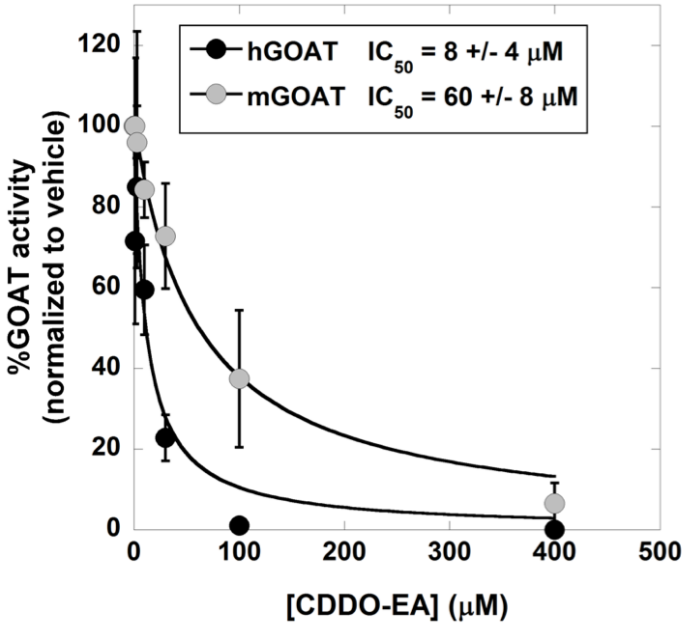

Figure 3.9 The mouse isoform of GOAT (mGOAT) is not as strongly inhibited by CDDO-EA as is hGOAT. Inhibition of hGOAT (black circles) and mGOAT (gray circles) as a function of CDDO-EA concentration. Error bars reflect the standard deviation of three independent measurements. Reprinted with permission from reference 1. © 2017, American Chemical Society.

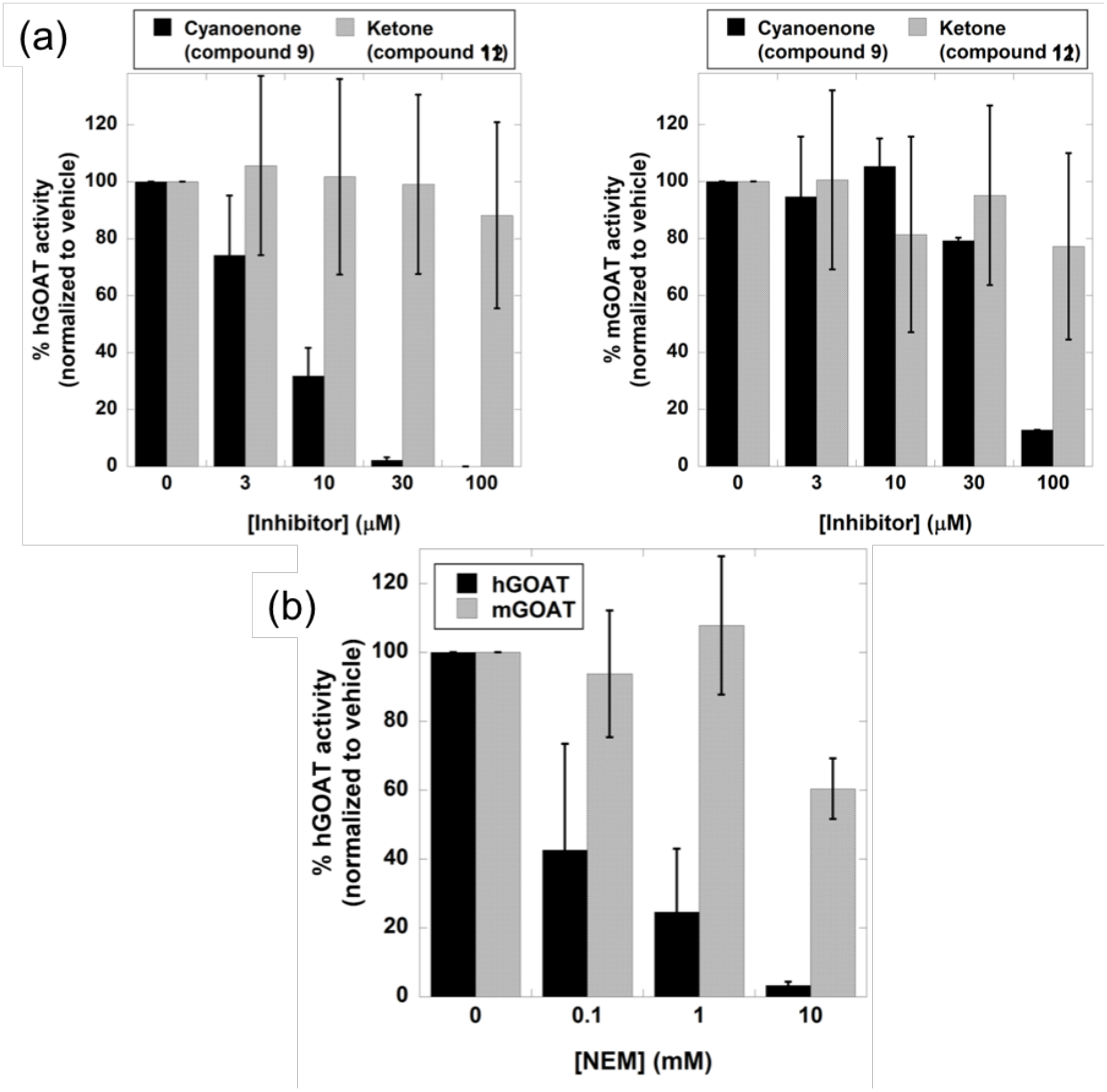

Figure 3.10 hGOAT and mGOAT exhibit dramatically different susceptibilities to inhibition by cysteine-modifying agents. (a) Inhibition by hGOAT (left) and mGOAT (right) octanoylation activity by α-cyanoenone **9** (black bars) and ketone **12** (gray bars). (b) Inhibition of hGOAT (black bars) and mGOAT (gray bars) octanoylation activity by *N*-ethylmaleimide (NEM). Error bars reflect the standard deviation from a minimum of three independent measurements. Reprinted with permission from reference 1. © 2017, American Chemical Society.

This resistance of mGOAT to inhibition was unexpected, as the two isoforms exhibit a high level of homology (79% identical and 92% similar) (Figure 3.11). In particular, residues required for enzyme activity are generally highly conserved, and the high degree of sequence conservation suggests the enzymes employ the same catalytic mechanism. Alignment of the hGOAT and mGOAT protein sequences reveals several cysteine residues in the human enzyme that are not conserved in the mouse isoform (Figure 3.11). This discrepancy in susceptibility to cysteine modifying compounds between these two closely related isoforms of GOAT suggests that either mGOAT does not contain a functionally essential cysteine, or that the functionally essential cysteine is resistant to modification by NEM and the cyanoenone inhibitors used in this work.

```
Homo_sapiens      MEWLWLFFLHPISFYQGAAFPFALLFNYLCIMDSFSTRARYLFLLTGGGALAVAAMGSYA  60
Mus_musculus      MDWLQLFFLHPLSFYQGAAFPFALLFNYLCILDTFSTRARYLFLLAGGGVLAFAAMGPYS  60
                  *:** ******:*******************:*:***********:***.**.**** *:

Homo_sapiens      VLVFTPAVCAVALLCSLAPQQVHRWTFCFQMSWQTLCHLGLHYTEYYLHEPPSVRFCITL  120
Mus_musculus      LLIFIPALCAVALVSFLSPQEVHRLTFFFQMGWQTLCHLGLHYTEYYLGEPPPVRFYITL  120
                  :*:* **:*****:. *:**:*** ** ***.*************** *** *** ***

Homo_sapiens      SSLMLLTQRVTSLSLDICEGKVKAASGGFRSRSSLSEHVCKALPYFSYLLFFPALLGGSL  180
Mus_musculus      SSLMLLTQRVTSLSLDICEGKVEAPRRGIRSKSSFSEHLWDALPHFSYLLFFPALLGGSL  180
                  **********************:*   *:**:**:***: .***:***************

Homo_sapiens      CSFQRFQARVQGSSALHPRHSFWALSWRGLQILGLECLNVAVSRVVDAGAGLTDCQQFEC  240
Mus_musculus      CSFRRFQACVQRSSSLYPSISFRALTWRGLQILGLECLKVALRSAVSAGAGLDDCQRLEC  240
                  ***:**** ** **:*:*  ** **:************:**:  .*.***** ***::**

Homo_sapiens      IYVVWTTAGLFKLTYYSHWILDDSLLHAAGFGPELGQSPGEEGYVPDADIWTLERTHRIS  300
Mus_musculus      IYLMWSTAWLFKLTYYSHWILDDSLLHAAGFGAEAGQGPGEEGYVPDVDIWTLETTHRIS  300
                  **::*:** ********************** * **.*********.****** *****

Homo_sapiens      VFSRKWNQSTARWLRRLVFQHSRAWPLLQTFAFSAWWHGLHPGQVFGFVCWAVMVEADYL  360
Mus_musculus      LFARQWNRSTALWLRRLVFRKSRRWPLLQTFAFSAWWHGLHPGQVFGFLCWSVMVKADYL  360
                  :*:*:**:*** *******::** ************************:**:***:****

Homo_sapiens      IHSFANEFIRSWPMRLFYRTLTWAHTQLIIAYIMLAVEVRSLSSLWLLCNSYNSVFPMVY  420
Mus_musculus      IHTFANVCIRSWPLRLLYRALTWAHTQLIIAYIMLAVEGRSLSSLCQLCCSYNSLFPVMY  420
                  **:***  *****:**:**:****************** ******  ** ****:**::*

Homo_sapiens      CILLLLLAKRKHKCN 435
Mus_musculus      GLLLFLLAERKDKRN 435
                   :**:***:**.* *
```

Figure 3.11 Clustal Omega alignment of hGOAT and mGOAT sequences. Cysteine residues are shown in bold, with cysteines conserved in both isoforms highlighted in gray, and cysteine residues unique to hGOAT highlighted in yellow. Reprinted with permission from reference 1.

3.3 Discussion and conclusions

Synthetic triterpenoids CDDO-Me and CDDO-EA, as well as their steroid-based counterpart compound **9**, represent a new class of hGOAT inhibitors. These molecules are only the second reported class of small molecule GOAT inhibitors, and the first reported inhibitors that do not bear a medium-length lipid chain mimicking the octanoyl group of the acyl donor substrate.[31, 32, 34, 38] Structure activity analysis of these compounds reveal a novel mechanism for hGOAT inhibition involving the covalent Michael addition of a nucleophilic cysteine residue to the α-cyanoenone, and inhibitor dilution experiments establish that inhibition by these groups is reversible. Surprisingly, steroids **10** and **11**, which contain a bromine and hydrogen at the α position, respectively, also displayed behavior consistent with reversible inhibition. The α protons in the enzyme-inhibitor conjugates resulting from covalent inhibition by these compounds are not expected to be acidic enough to enable a reversible Michael reaction. One possible explanation for the observed behavior is that these inhibitors modify a cysteine residue within the active site of hGOAT, blocking activity, and the proposed general base (such as the conserved and functionally required H338[49-51]) catalyzes the reverse Michael reaction. However, in the case of compound **11**, there is a dramatic difference between observed activity under the undiluted and diluted reaction conditions, with the undiluted reaction – containing 600 μM inhibitor – resulting in no or minimal enzyme activity, behavior which is not seen with other compounds with lower IC_{50} values. This nearly complete loss of activity under these reaction conditions may reflect issues of inhibitor solubility, rather than true enzyme inhibition. Looking forward, steroid compounds featuring a thioether adduct at the β position may enable the direct monitoring of the reverse Michael reaction.

Treatment of HEK293FT cells expressing mGOAT and preproghrelin with CDDO-EA and CDDO-Me resulted in reduced concentrations of acyl ghrelin. However, enzymatic assays

comparing the mouse and human isoforms of GOAT revealed that mGOAT is less susceptible to inhibition by these synthetic triterpenoids, and does not exhibit chemoselectivity for inhibition by cysteine-modifying compounds. Therefore, the reduced concentrations of acyl ghrelin upon treatment with CDDO derivatives in cell-based assays likely resulted, at least in part, from mechanisms aside from GOAT inhibition. Total ghrelin (acyl + unacylated) concentrations could not be determined due to unreliability of the commercial ELISA kit for total ghrelin. CDDO derivatives have been shown to modulate cytoprotective and apoptotic genes regulated through the Nrf2 and NF-κB transcription factors,[41] and it is possible that treatment with these compounds reduced expression of ghrelin and/or GOAT, resulting in the observed decreased concentrations of acyl ghrelin.

Confirmation that synthetic triterpenoids inhibit GOAT in cells will rely on the development of a cellular assay using hGOAT and a method for reliably determining concentrations of total ghrelin. However, clinical and preclinical trials of CDDO derivatives have reported side effects that may be consistent with altered ghrelin signaling, including reduced fat deposition, improved glucose tolerance, prevention of insulin resistance, and weight loss.[52-55] Future studies investigating the effects of these molecules in either animal models or human patients should incorporate the monitoring of ghrelin levels to establish whether GOAT is a true target (or off-target) or these drugs *in vivo*.

Aside from the therapeutic implications of this study, inhibition by synthetic triterpenoids also provided fundamental information about GOAT. Structure-activity analysis of the steroid-based inhibitors and inhibition by NEM established that hGOAT catalysis may involve a functionally essential cysteine. There are 16 cysteine residues within hGOAT, with the majority of them located outside the conserved C-terminal "MBOAT" domain.[56] Mutational analyses of

GOAT, Hhat, and PORCN have revealed functionally essential residues, but none have implicated cysteine residues as being functionally essential.[31, 46, 49, 51, 57-60] The specific role of the modified cysteine residue(s) is unclear. If the cysteine responsible for hGOAT inhibition by these compounds is in fact catalytic, it may act by forming an acyl-enzyme intermediate, transferring the octanoyl group from octanoyl CoA to ghrelin (Figure 3.12). A similar mechanism has been proposed for protein palmitoylation by DHHC palmitoyl transferases.[61, 62] The resistance of mGOAT to inhibition by cysteine-modifying molecules argues that the cysteine responsible for inhibition of hGOAT is not catalytic. In this case, the cysteine residue within hGOAT may be located in or near the active site, so that modification by inhibitors of this residue sterically blocks the active site. Given either possibility, the cysteine residues(s) involved in inhibition by these inhibitors may serve as a chemical handle to determine the enzyme's catalytic machinery.

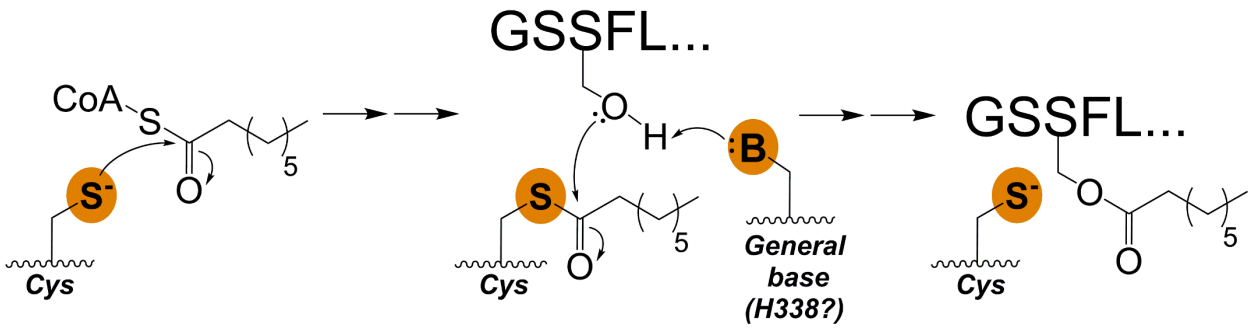

Figure 3.12 Potential mechanism for hGOAT-catalyzed ghrelin octanoylation employing a cysteine acyl-enzyme intermediate. Following formation of an octanoyl-enzyme intermediate, transfer of the octanoyl group to the serine acylation site near the N-terminus of ghrelin can be catalyzed through the involvement of a general base such as the conserved and functionally essential H338 residue. Reprinted with permission from reference 1. © 2017, American Chemical Society.

3.4. Materials and methods

General

Data plotting and curve fitting were performed with Kaleidagraph (Synergy Software). Methoxy arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical (Ann Arbor, MI) as a stock in methyl acetate and diluted in DMSO prior to use. Octanoyl coenzyme A (octanoyl CoA) was purchased from Advent Bio (Downers Grove, IL) solubilized to 5 mM in 10 mM Tris-HCl (pH 7.0), aliquoted into low-adhesion microcentrifuge tubes, and stored at -80 °C. Acrylodan (Anaspec) was solubilized in acetonitrile, with the stock concentration determined by absorbance at 393 nm upon dilution into methanol (ε = 18,483 M^{-1} cm^{-1} per manufacturer's data sheet). *N*-ethylmaleimide (NEM) was purchased from Tokyo Chemical Industry (Portland, OR), and dissolved in ethanol prior to use. CDDO-Me, CDDO-EA, CDDO-Im, CDDO-TFEA, and CDDO were gifts from G. Gribble and M. Sporn (Dartmouth College, Hanover, NH). Compounds **6-8** and **15** were obtained from the Developmental Therapeutics Program (DTP) of the National Institutes of Health (NIH). Compounds **9-14, 16,** and **17** were synthesized by Nivedita Mahajani as described below. Estrone (**15**) was purchased from Cayman Chemical. Cyclohexenone (**18**) was purchased from Alfa Aesar (Ward Hill, MA). The $GSSFLC_{NH2}$ peptide for fluorescent labeling with acrylodan was synthesized by Sigma-Genosys in the Pep-screen format, and solubilized in 1:1 acetonitrile: H_2O and stored at -80 °C. Peptide concentration was determined by absorbance at 412 nm following reaction of the cysteine thiol with 5,5'-dithiobis(2-nitrobenzoic acid) using ε = 14,150 M^{-1} cm^{-1}.[63]

Expression and enrichment of hGOAT and mGOAT

Genes encoding hGOAT or mGOAT with a C-terminal triple tag (FLAG, HA, His_6) were cloned into the pFastBacDual vector (Invitrogen) using *Eco*RI and *Xba*I restriction sites, with the resulting vector used to produce the baculovirus expression system protocol (Invitrogen). Sf9 insect cells (5.0×10^8 cells in a 500 mL total culture volume) were infected with hGOAT baculovirus at a multiplicity of infection of 10 followed by protein expression for 40 hr at 28 °C with shaking at 150 rpm. Cells were harvested by centrifugation (500 x *g*, 5 min), and freezing the pellet at -80 °C. The cell pellets were thawed on ice, resuspended in 25 mL lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.0), 1 mM sodium ethylenediamine tetraacetate (NaEDTA), 1 mM dithiothreitol (DTT), complete protease inhibitor (Roche), 10 μg/mL pepstatin A, and 100 μM bis(4-nitrophenyl)phosphate]. The resuspended cells were lysed with a Dounce homogenizer on ice, followed by removal of cell debris by centrifugation (3,000 x *g*, 4 °C, 10 min). The microsomal fraction was then isolated by ultracentrifugation of the supernatant (100,000 x *g*, 4 °C, 1 hr). The isolated microsomal fraction pellet was resuspended in 50 mM HEPES (pH 7.0) and stored in low-adhesion microcentrifuge tubes at -80 °C until use.

Peptide substrate fluorescent labeling

$GSSFLC_{NH2}$ (300 μM) and acrylodan (500 μM) were dissolved in 500 μL 1:1 50 mM HEPES (pH 7.8):acetonitrile, followed by incubation at room temperature in the dark for 18 hr with shaking. Acrylodan-labeled peptide was purified by reverse phase HPLC (Zorbax Eclipse XDB column, 9.4 x 250 mm) using an isocratic mobile phase of water containing 0.05% trifluoroacetic acid (TFA) (65%) and acetonitrile (35%) flowing at 4.2 mL/min over 21 min). Labeled peptide eluted around 8 min, monitoring UV absorbance at 360 nm. Collected fractions

containing labeled peptides were dried under vacuum at room temperature and resuspended in 1:1 acetonitrile:H_2O, and labeling was confirmed by MALDI-TOF mass spectrometry (Bruker Autoflex III, SUNY-ESF) using a matrix of saturated sinapinic acid in 0.1% TFA and 50 mM ammonium phosphate. Concentration of acrylodanylated peptide was determined by absorbance of acrylodan at 360 nm (ε = 13,300 M^{-1} cm^{-1}), and was stored at -80 °C.

hGOAT and mGOAT activity assays and analysis

Microsomal fraction from Sf9 cells expressing hGOAT or mGOAT were thawed on ice and passed through an 18-gauge needle 10 times to homogenize the fraction. Assays were performed with ~100 μg of microsomal protein, as determined by a Bradford assay. The microsomal fraction was preincubated with 1 μM MAFP and inhibitor or vehicle as indicated in 50 mM HEPES (pH 7.0) for 30 min at room temperature. Reactions were initiated with the addition of 500 μM octanoyl CoA and 1.5 μM fluorescently labeled ghrelin mimetic $GSSFLC_{AcDan}$ in a total volume of 50 μL, and were incubated for 3 hr, then stopped with the addition of 50 μL of 20% acetic acid in isopropanol, and solutions were clarified by protein precipitation with 16.7 μL of 20% trichloroacetic acid (TCA) followed by centrifugation (1,000 x *g*, ~1 min). The supernatant was analyzed by reverse phase HPLC. Data reported are the average of three independent determinations, with error bars representing one standard deviation.

Assay samples were analyzed on an Agilent 1260 HPLC with a C18 reverse phase column (Zorbax Eclipse, 4.6 x 150 mm) using a gradient of 30% acetonitrile in 0.05% TFA to 63% acetonitrile in 0.05% TFA over 14 min, followed by 100% acetonitrile for 10 min. Fluorescent peptides were detected by fluorescence (λ_{ex} = 360 nm, λ_{em} = 485 nm), with the unacylated peptide eluting with a retention time of ~6 min and the octanoylated peptide product eluting with a

retention time of ~12 min. Chromatogram analysis and peak integration was performed using Chemstation for LC (Agilent Technologies).

Library screening

For screening of the Diversity Set IV library of small molecules, hGOAT octanoylation reactions were performed as described above with the addition of library compounds at concentrations of 10 and 100 μM. Compounds that met the criteria for inhibition (dose-dependent decrease in activity, <50% activity at 100 μM) were confirmed with a secondary screen using the same protocol.

Determination of IC_{50} values in in vitro *hGOAT and mGOAT activity assays*

Reactions were performed and analyzed as described in the presence of either inhibitor or vehicle as appropriate. The percent activity at each inhibitor concentration was calculated from HPLC integration data using equations 1 and 2:

$$(1) \qquad \%\ \text{activity} = \frac{\%\ \text{peptide octanoylation in the presence of inhibitor}}{\%\ \text{peptide octanoylation in the absence of inhibitor}}$$

$$(2) \qquad \%\ \text{peptide octanoylation} = \frac{\text{integrated fluorescence of octanoylated peptide}}{\text{total peptide integrated fluorescence (octanoylated and non-octanoylated)}}$$

To determine the IC_{50} value, the plot of % activity versus [inhibitor] was fit to equation 3, with % activity_0 denoting hGOAT activity in the presence of vehicle alone:

$$(3) \qquad \%\ \text{activity} = \%\text{activity}_0\left(1 - \frac{[\text{inhibitor}]}{[\text{inhibitor}] + IC_{50}}\right)$$

Determination of inhibitor time dependence with hGOAT

Assays were performed and analyzed as described above with the following modifications. The microsomal fraction was preincubated with 1 μM MAFP in 50 mM HEPES (pH 7.0) for 30 min at room temperature, and incubated with inhibitor or vehicle as appropriate for varying times (5, 10, 30, and 60 min) prior to initiation of the reaction. Reactions were incubated at 10 min at room temperature in the dark, followed by addition of stop solution and reaction workup and analysis as described above.

Inhibitor reversibility assay

The undiluted homogenized microsomal fraction containing hGOAT (protein concentration of ~7 μg/μL) was incubated with 10 μM MAFP and 3 x (IC_{50}) or equal volume vehicle for 30 min at room temperature. The microsomal fraction/inhibitor solution was diluted 10-fold into a reaction mixture containing 500 μM octanoyl CoA, 1.5 μM $GSSFLC_{AcDan}$, 50 mM HEPES (pH 7.0.), and either vehicle or inhibitor [final concentration of 3 x (IC_{50})] in a total reaction volume of 50 μL. Reaction mixtures were incubated at 3 hours at room temperature in the dark and analyzed as described above.

Cell line generation and culture

Stably transfected GOAT/preproghrelin HEK 293FT cells were generated using the phPPG-mGOAT plasmid (a gift from Dr. Jef Boeke's laboratory, NYU Langone Medical Center) containing human preproghrelin (hPPG) and mouse ghrelin *O*-acyltransferase (mGOAT) connected by an intervening encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) under puromycin selection as previously described.[34] Cells were transfected using

Lipofectamine 2000 (Invitrogen) at a 2 μL to 1 μg DNA ratio in 10 cm tissue culture-treated dishes according to manufacturer's protocol. The 293FT-hPPG-mGOAT cells were cultured in DMEM medium (Corning) supplemented with 10% inactivated fetal bovine serum, 1% penicillin/streptomycin, 1 μg/mL puromycin, and 0.01% octanoic acid and routinely passaged 2-3 times per week. A clonal line was chosen through serial dilution into a 96-well plate.

Inhibitor treatment, cell lysate preparation, and acyl ghrelin concentration measurement by ELISA

Cell density and number were optimized for detection of acyl ghrelin via ELISA (EMD Millipore Corp). HEK293FT-hPPG-mGOAT clonal cells were plated in 24-plates at 2 x 10^5 cells/well. Cells were incubated overnight at 37 °C, 5% CO_2 and treated the next morning with inhibitors or vehicle (DMSO) in triplicate. Cells were treated for 24 hr and harvested after medium aspiration using 40 μL/well trypsin-EDTA (0.25%, Corning) containing 5 μM methoxy arachidonyl fluorophosphonate (MAFP). Cells were transferred to 1.5 mL siliconized tubes with 200 μL PBS containing 5 μM MAFP and centrifuged at 800 x *g* for 5 min. The pellet was resuspended in 100 μL lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, Complete Protease Inhibitor Tab (Roche), 5 μM MAFP), vortexed for 3 seconds on high and frozen at -80 °C for a minimum of 16 hr. Immediately before use, lysates were thawed on ice, centrifuged at 20,000 x *g* for 20 min at 4 °C, and the supernatant was transferred to another 1.5 mL siliconized tube. Lysate was diluted 1:2 with lysis buffer before loading onto Human Active Ghrelin ELISA plates (EMD Millipore Corp). Samples were incubated with the capture and detection antibodies for 3.5 hr shaking at room temperature, with all other steps performed according to manufacturer's protocol.

Determination of inhibitor cytotoxicity by AlamarBlue assay

HEK293FT-hPPG-mGOAT cells were plated into 96-well plates at 2 x 10^4 cells/well the evening before inhibitor addition. Cells were treated with inhibitor and 10% (v/v) AlamarBlue (Invitrogen) for 24 hr, followed by determination of cell viability according to the manufacturer's protocol using a Synergy H1 Hybrid plate reader (BioTek).

General information for synthetic methods (Syntheses performed by Nivedita Mahajani, Syracuse University)

All anhydrous reactions were run under a positive pressure of argon or nitrogen. All syringes, needles, and reaction flasks required for anhydrous reactions were dried in an oven and cooled under an N_2 atmosphere or in a desiccator. DCM and THF were dried by passage through an alumina column by the method of Grubbs.[64] Triethylamine was distilled from CaH_2. All other reagents and solvents were purchased from commercial sources and used without further purification

Analytical thin layer chromatography (TLC) was performed on precoated glass backed plates (silica gel 60 F254; 0.25 mm thickness). The TLC plates were visualized by UV illumination and by staining. Solvents for chromatography are listed as volume:volume ratios. Flash column chromatography was carried out on silica gel (40-63 μm). Melting points were recorded using an electrothermal melting point apparatus and are uncorrected. Elemental analyses were performed on an elemental analyzer with a thermal conductivity detector and 2 meter GC column maintained at 50 °C.

Ketone **12**,[65] α-bromoketone **13**,[66, 67] enone **14**,[68] 2-cyano-2-cyclohexanone **16**,[69] and 2-bromo-2-cyclohexanone **17**[70] were prepared and purified as previously described. Estrone **15** and 2-cyclohexen-1-one **18** were purchased from commercial sources.

Identity (performed by Nivedita Mahajani, Syracuse University)

Proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were recorded at 300 or 400 MHz and 75 or 100 MHz respectively. The chemical shifts are given in parts per million (ppm) on the delta (δ) scale. Coupling constants are reported in hertz (Hz). The spectra were recorded in solutions of deuterated chloroform ($CDCl_3$), with residual chloroform (δ 7.26 ppm for ^{1}H NMR, δ 77.23 ppm for ^{13}C NMR) or tetramethylsilane (δ 0.00 for ^{1}H NMR, δ 0.00 for ^{13}C NMR) as the internal reference. Data are reported as follows: (s = singlet; d = doublet; t = triplet; q = quartet; p = pentet; sep = septet; dd = doublet of doublets; dt = doublet of triplets; td = triplet of doublets; tt = triplet of triplets; qd = quartet of doublets; ddd = doublet of doublet of doublets; br s = broad singlet). ^{1}H and ^{13}C NMR spectra for compounds **9-11** are provided in Figure 13. Infrared (IR) spectra were obtained as thin films on NaCl plates by dissolving the compound in DCM followed by evaporation or as KBr pellets.

*Synthesis of (5S,8S,9S,10S,13S,14S)-10,13-dimethyl-3-oxo-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthrene-2-carbonitrile (compound **9**). (Synthesis performed by Nivedita Mahajani, Syracuse University)*

To a stirred solution of bromo enone **10** (0.210 g, 0.597 mmol) in anhydrous DMF (5.9 mL) was added copper (I) cyanide (0.059 g, 0.657 mmol) and potassium iodide (0.020 g, 0.2 mmol). The resulting reaction mixture was heated to 120 °C for 36 hr. After the completion of reaction, it was cooled to room temperature, quenched with water (5 mL), and diluted with ethyl

acetate (15 mL). The organic phase was washed with saturated $NaHCO_3$ (2 x 5 mL) and brine (5 mL), dried over sodium sulfate, filtered and concentrated. Silica gel column chromatography on the residue (8% ethyl acetate/92% hexanes) provided 0.070 g (40%) of cyano enone **9** as white solid. This procedure was adapted from the work of Gribble.[71]

mp = 171-173 °C; TLC R_f = 0.61 (20% ethyl acetate/80% hexanes); IR (thin film from DCM) 3411, 2230, 1693, 1447, 1216 cm^{-1}; $[\alpha]_D^{25}$ = +1.46 (c = 0.05, DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (s, 1H), 2.47-2.34 (m, 2H), 2.00-1.92 (m, 1H), 1.84-1.54 (m, 7H), 1.50-1.38 (m, 5H), 1.22- 1.12 (m, 3H), 1.08 (s, 3H), 1.05-0.94 (m, 2H), 0.73 (s, 3H); ^{13}C NMR (100 MHz, CDCl3) δ 192.4, 169.8, 115.8, 114.5, 54.3, 49.4, 43.2, 40.9, 40.1, 38.4, 35.9, 35.8, 31.3, 27.3, 25.3, 22.6, 21.2, 20.5, 17.6, 12.6. HRMS (ESI+) calculated for $C_{20}H_{27}NONa$: 320.1984, Found: 320.1981. Anal. calculated for $C_{20}H_{27}NO$: C, 80.76; H, 9.15; N, 4.71. Found: C, 80.37; H, 9.13; N, 4.48.

*(5S,8S,9S,10S,13S,14S)-10,13-dimethyl-3-oxo-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthrene-2-bromide (compound **10**). (Synthesis performed by Nivedita Mahajani, Syracuse University)*

To a solution of cyclohexenone **11** (0.100 g, 0.367 mmol) in DCM (1 mL) under argon at 0 °C, a solution of bromine (0.019 mL, 0.367 mmol) in DCM (1 mL) was added dropwise over 15 min. Triethylamine (0.087 mL, 0.623 mmol) was added and the resulting mixture was allowed to warm to room temperature and stirred for another 1.5 hr before it was quenched with 1 M HCl. The layers were separated and the organic layer was washed twice with sodium thiosulfate, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified

by silica gel column chromatography (2% ethyl acetate/98% hexane), which afforded bromo enone **10** (0.103 g, 80%) as a white solid.

mp = 111-116 °C ; TLC R_f = 0.52 (10% ethyl acetate/90% hexanes); IR (thin film from DCM) 2964, 2848, 1691, 1436, 954, 755 cm^{-1}; $[\alpha]_D^{25}$ = +22.3 (c = 0.50, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.60 (s, 1H), 2.56-2.43 (m, 2H), 2.04-1.96 (m, 1H), 1.83-1.57 (m, 6H), 1.48-1.34 (m, 5H), 1.22-1.12 (m, 3H), 1.05 (s, 3H), 1.02-0.93 (m, 3H), 0.73 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.5, 158.9, 123.1, 54.4, 50.2, 44.2, 42.8, 41.0, 40.9, 40.2, 38.5, 35.7, 31.5, 27.2, 25.4, 21.4, 20.5, 17.6, 12.9. HRMS (ESI+) calculated for $C_{19}H_{28}BrONa$: 373.1137, Found: 373.1135. Anal. calculated for $C_{19}H_{27}BrO$: C, 64.96; H, 7.75. Found: C, 64.72; H, 7.94.

*(2R,5S,8S,9S,10S,13S,14S)–10,13–dimethyl–4,5,6,7,8,9,10,11,12,13,14,15,16,17–tetradecahydro–3H–cyclopenta[a]phenanthren–3–one (compound **11**). (Synthesis performed by Nivedita Mahajani, Syracuse University)*

A suspension of α-bromoketone **13** (0.600 g, 1.70 mmol), lithium bromide (0.884 g, 10.2 mmol), and lithium carbonate (0.752 g, 10.2 mmol) in DMF (6.6 mL) was heated to 80 °C. After ~18 hr the reaction mixture was cooled to room temperature and then poured over crushed ice. The quenched reaction mixture was extracted with ethyl acetate (3 x 20 mL). The organic layers were collected, combined, washed with cold water and brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified using silica gel column chromatography (2% ethyl acetate/98% hexane), which afforded enone **11** (0.371 g, 80%) as a white solid.

TLC R_f = 0.52 (10% ethyl acetate/90% hexane); $[\alpha]_D^{25}$ = +32.8 (c = 0.87, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.13 (d, J = 10.2 Hz, 1H), 5.82 (dd, J = 10.2, 0.9 Hz, 1H), 2.34 (dd, J = 13.2,

10.6 Hz, 1H), 2.21 (ddd, J = 17.6, 4.1, 0.9 Hz, 1H), 1.93–1.85 (m, 1H), 1.79–1.69 (m, 3H), 1.66–1.51 (m, 3H), 1.49–1.33 (m, 5H), 1.18–1.10 (m, 3H), 1.02–0.90 (m, 6H), 0.71 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.2, 158.6, 127.4, 54.5, 50.2, 44.3, 41.0, 40.9, 40.3, 39.1, 38.7, 36.1, 31.7, 27.7, 25.4, 21.3, 20.5, 17.6, 13.0. HRMS (ESI+) calculated for $C_{19}H_{28}ONa$: 295.2032, Found: 295.2030. Anal. calculated for $C_{19}H_{28}O$: C, 83.77; H, 10.36; Found: C, 83.83; H, 10.49.

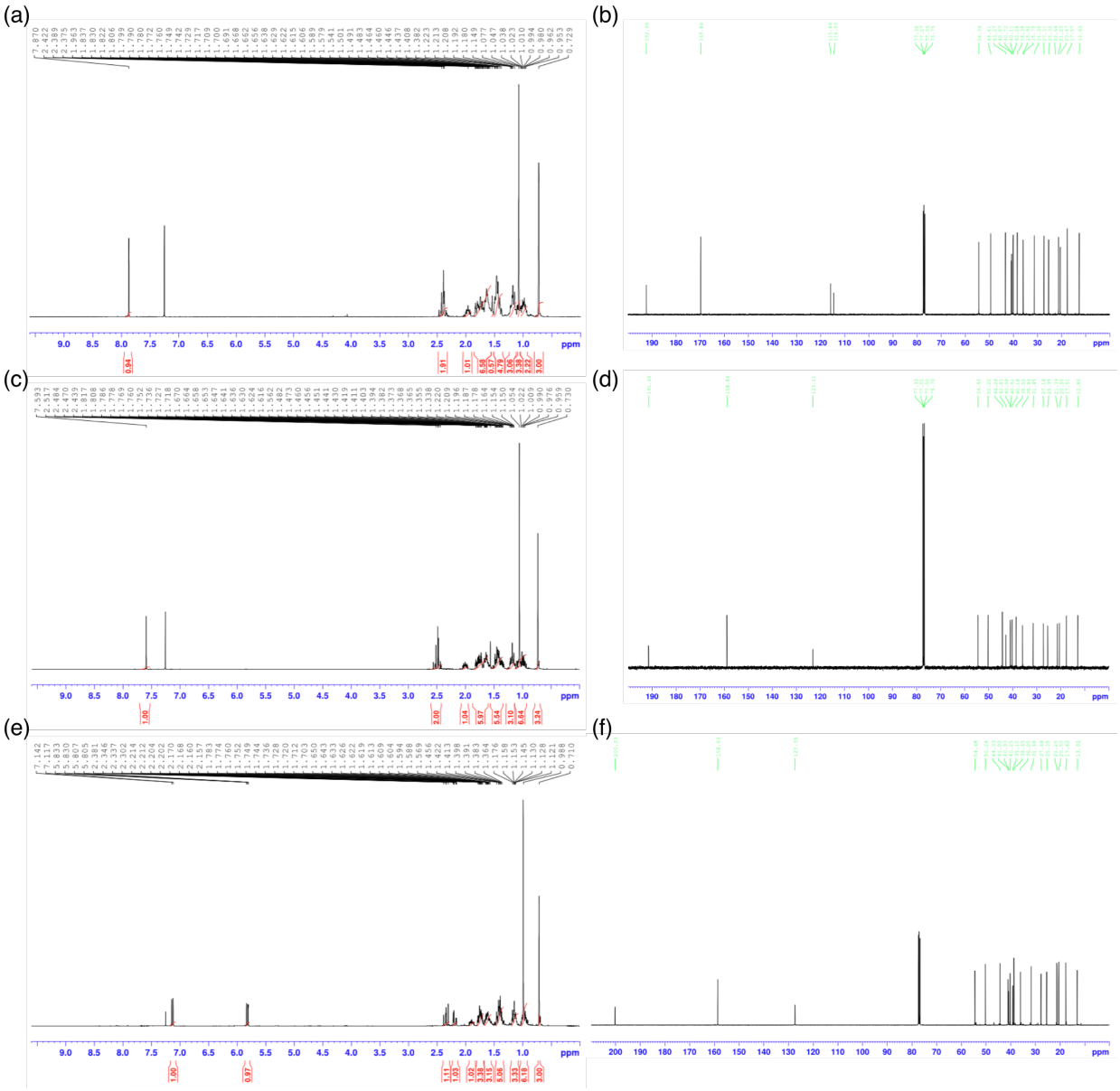

Figure 3.13 ^{1}H and ^{13}C NMR spectra for compounds **9-11**. (a) ^{1}H NMR spectrum and (b) ^{13}C NMR spectrum for compound **9**. (c) ^{1}H NMR spectrum and (d) ^{13}C NMR spectrum for compound **10**. (e) ^{1}H NMR spectrum and (f) ^{13}C NMR spectrum for compound **11**. Reprinted with permission from reference 1. © 2017, American Chemical Society.

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Chapter 4: Mutagenesis studies to locate functionally essential residues within hGOAT

Initial design and creation of hGOAT mutant constructs was performed in collaboration with Rosemary Loftus, a Hougland research group Master's student. Cloning of hGOAT_ΔC_1-4, hGOAT_ΔC_1-5/6, hGOAT_ΔC_1-5/6,9-11, and hGOAT_ΔC_9-11 into the pFastBacDual vector and activity screening and expression analysis of the resulting enzymes were performed in part by Michael Aiduk.

4.1 Introduction

Despite ongoing studies since its discovery in 2008,[1, 2] many aspects of GOAT structure and function remain undefined. As indicated by its initial assignment as the fourth member of the MBOAT enzyme superfamily, the sequence of GOAT reveals homology with other MBOAT family members with the C-terminus of GOAT comprising the conserved "MBOAT domain."[3] Two residues within this domain are highly conserved across MBOAT family members and potentially implicated in catalysis: asparagine 307 (N307), which is highly conserved, and histidine 338 (H338), which is absolutely conserved.[3] Mutation of either of these residues within GOAT abolishes activity.[1, 2] Mutation of the conserved asparagine/aspartate residue results in a complete loss of activity in Hhat,[4, 5] but has been shown to have no effect on PORCN activity.[6, 7] Conversely, mutation of the conserved histidine results in complete loss of activity in PORCN,[6-8] but only a moderate reduction of activity in Hhat.[4, 5, 9] Although H338 and N307 are implicated in GOAT catalysis based on conservation and mutational analysis, the active site and catalytic mechanism of GOAT remain undefined.

As an integral membrane protein, GOAT has proven resistant to purification protocols that maintain enzymatic activity, preventing structural studies of the enzyme.[10] In 2013, Taylor and coworkers determined GOAT's structural topology within the ER membrane using selective permeabilization and epitope tag detection.[11] Bioinformatics analysis predicted GOAT contains 12 transmembrane helices. Using mouse GOAT constructs containing N-terminal, C-terminal, or internal epitope tags placed between the predicted helices followed by selective membrane permeabilization, the loops between helices were assigned to either the lumen of the endoplasmic reticulum or the cytoplasm. The final topology of GOAT was determined to contain 11 transmembrane helices and one re-entrant loop, with the N-terminus residing in the lumen and the

C-terminus in the cytoplasm. Interestingly, this topology predicted that N307 and H338 were located on opposite sides of the membrane, indicating that both could not be directly involved in the GOAT active site.

After the publication of GOAT's topology, the topology of Hhat was reported by two independent groups.[5, 12] Using selective permeabilization and immunofluorescence, the C-terminal two-thirds of Hhat was shown to have remarkably similar topology to that of GOAT, with the invariant histidine residing in the lumen and the conserved aspartate (equivalent to N307 in GOAT) residing in a cytosolic loop,[5, 12] providing further support of this topological model as an important feature of the MBOAT family enzymes.

In the absence of structural information, mutagenesis of specific residues within GOAT can provide information about the enzyme's requirements for ghrelin acylation. Using the fact that a serine hydroxyl within ghrelin is acylated as a guide for our mutagenesis campaign, we evaluated potential mechanisms GOAT may employ to catalyze this acylation, based on known mechanisms of other enzymes. We hypothesize GOAT employs a general base to activate the serine 3 hydroxyl of ghrelin to act as a nucleophile. Classic serine proteases such as chymotrypsin or subtilisin contain a catalytic triad in which a serine hydroxyl acts as the nucleophile and a histidine acts as a general base to activate the serine, with an aspartate stabilizing the conjugate acid of histidine.[13] Glycerol phosphate acyltransferases (GPATs) contain a conserved HX_4D motif,[14] with the histidine and aspartate forming an ion pair to activate a hydroxyl of the glycerol 3-phosphate substrate.[15] In addition to aspartate (D) and histidine (H), enzymes can also use glutamate (E) or a metal-bound water molecule/hydroxyl as a general base, with catalytic metals most frequently coordinated by cysteine (C), aspartate (D), glutamate (E), or histidine (H).

To identify functionally essential residues within GOAT, hGOAT variants containing single-site mutations were designed and expressed. Residues for mutation were selected based on the following criteria: 1) the residue can act as a general base or metal ligand (H, D, E, or C); 2) the residue was within the conserved C-terminal MBOAT domain of the enzyme; and 3) the residue was conserved across multiple species. Fifteen residues within hGOAT met this criteria (Figure 4.1), and primers were designed to mutate these positions to either alanine (A) or a structurally conservative residue (phenylalanine, F, for H; asparagine, N, for D; glutamine, Q, for E; and serine, S, for C). Although it does not strictly meet these criteria, the highly conserved N307 was also mutated. These variants were expressed and screened for activity to determine which of these sixteen residues were required for ghrelin acylation.

As described in Chapter 3, there is recent evidence that hGOAT contains at least one functionally required cysteine residue based on inhibition by NEM and synthetic triterpenoids.[16] Therefore, in addition to mutations targeting potential general bases, we also developed a series of hGOAT mutant variants to determine the role of each of the sixteen cysteine residues within hGOAT. A summary of this work is included herein, with complete characterization of the hGOAT cysteine mutants an ongoing research project within the Hougland laboratory.

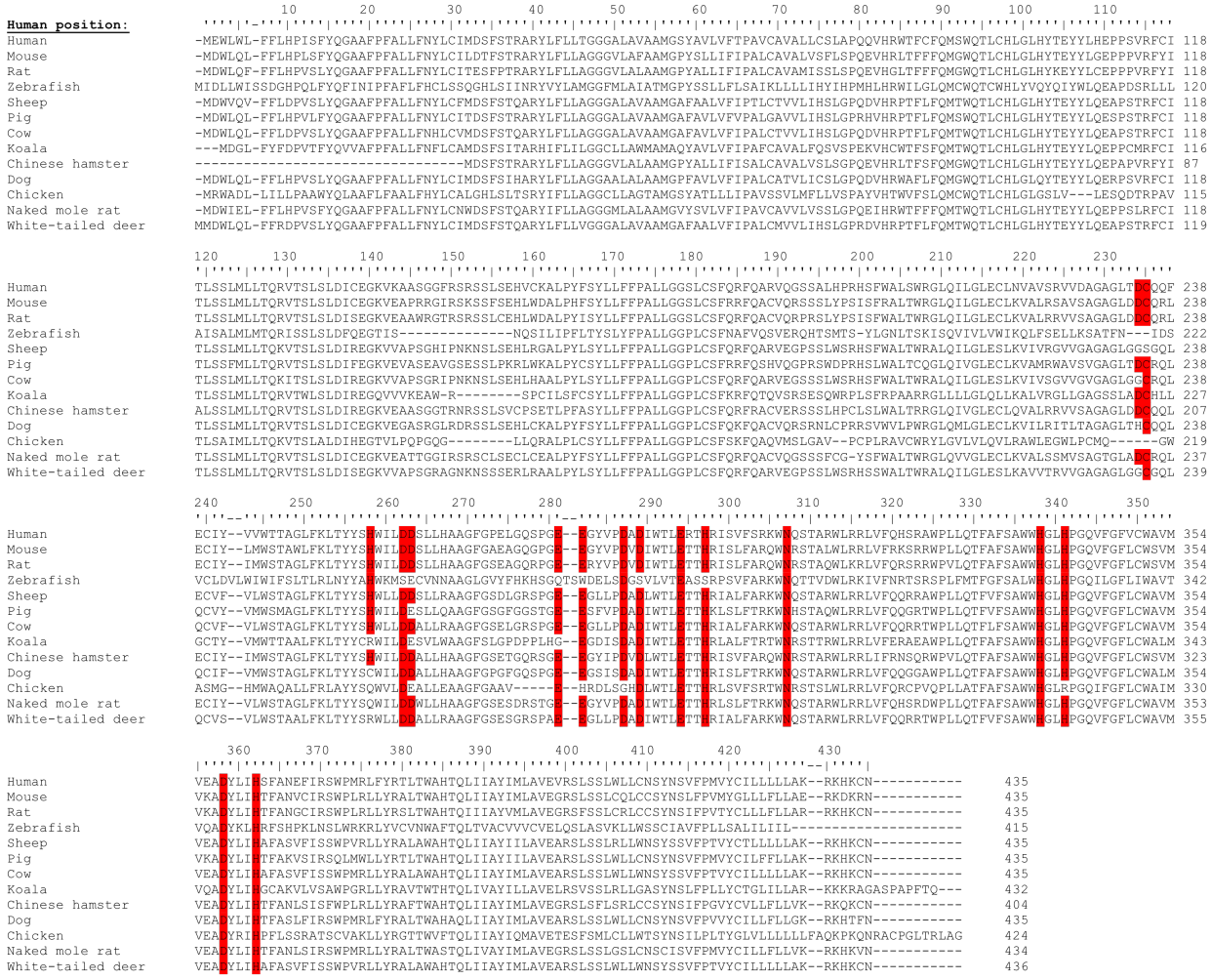

Figure 4.1 Clustal Omega alignment of GOAT sequences from multiple species. Residues selected for mutagenesis within hGOAT are highlighted in red.

4.2 Results

Mutations targeting potential catalytic bases

Sixteen residues within hGOAT were selected for mutagenesis (Figure 4.1). Mutagenesis primers were designed to mutate each position to alanine (Table 4.1), resulting in sixteen variants of hGOAT-HA-FLAG-His_6 within the pFastBacDual vector. The hGOAT gene was transposed into bacmid DNA using the Bac-to-Bac expression system (Invitrogen). Purified bacmid was transfected into Sf9 insect cells to generate baculovirus, which was used to infect Sf9 cells for expression of hGOAT. Expressed hGOAT variants were obtained as enriched microsomal fraction from insect cells through fractional ultracentrifugation. Expression of each hGOAT variant containing a single-site alanine mutation was determined by SDS-PAGE and immunoblotting (Western blotting) against the FLAG epitope tag. Successful hGOAT-HA-FLAG-His_6 expression was supported by the presence of a band at ~55 kDa, which was observed for all alanine mutants except for E281A, E282A, and D289A (Figure 4.2).

Activity of each mutant variant was determined using a fluorescently-labeled ghrelin mimetic substrate, $GSSFLC_{AcDan}$, monitoring octanoylation by an increase in retention time on reverse-phase HPLC.[17] Mutation of seven residues to alanine resulted in complete loss of observable activity (Figure 4.3). Of these seven inactive variants, expression of E282A was not confirmed by Western blot, leaving open the possibility that the lack of activity arises from lack of enzyme expression rather than loss of a functionally required residue. A peak at 12.5 minutes, corresponding to the octanoylated peptide,[17] was observed for the H258A, D262A, E281A, D287A, D289A, E294A, H297A, H341A, and H362A mutants, indicating that these residues are not functionally required for catalysis (Figure 4.3).

D234A	Forward: 5’-GCCGGACTGACTGCTTGCCAGCAATTCGAATG-3’ Reverse: 5'-CATTCGAATTGCTGGCAAGCAGTCAGTCCGGC-3'
C235A	Forward: 5'-GGAGCCGGACTGACTGATGCCCAGCAATTCGAATG-3' Reverse: 5'-CATTCGAATTGCTGGGCATCAGTCAGTCCGGCTCC-3'
H258A	Forward: 5'-GCTGACCTACTACTCAGCCTGGATCCTCGACG-3' Reverse: 5'-CGTCGAGGATCCAGGCTGAGTAGTAGGTCAGC-3'
D262A	Forward: 5'-CACACTGGATCCTCGCCGATTCGCTCTTGC-3' Reverse: 5'-GCAAGAGCGAATCGGCGAGGATCCAGTGTG-3'
D263A	Forward: 5'-CTGGATCCTCGACGCTTCGCTCTTGCACG-3' Reverse: 5'-CGTGCAAGAGCGAAGCGTCGAGGATCCAG-3'
E281A	Forward: 5'-GACAGTCACCAGGAGCGGAAGGTTACGTTCC-3' Reverse: 5'-GGAACGTAACCTTCCGCTCCTGGTGACTGTC-3'
E282A	Forward: 5'-GTCACCAGGAGAGGCAGGTTACGTTCCTG-3' Reverse: 5'-CAGGAACGTAACCTGCCTCTCCTGGTGAC-3'
D287A	Forward: 5'-GGTTACGTTCCTGCCGCTGATATCTGGACC -3' Reverse: 5'-GGTCCAGATATCAGCGGCAGGAACGTAACC-3'
D289A	Forward: 5'-CGTTCCTGACGCTGCTATCTGGACCCTGG-3' Reverse: 5'-CCAGGGTCCAGATAGCAGCGTCAGGAACG-3'
E294A	Forward: 5'-GATATCTGGACCCTGGCAAGGACTCACAGAATC-3' Reverse: 5'-GATTCTGTGAGTCCTTGCCAGGGTCCAGATATC-3'
H297A	Forward: 5'-CCCTGGAAAGGACTGCCAGAATCTCGGTCTTC-3' Reverse: 5'-GAAGACCGAGATTCTGGCAGTCCTTTCCAGGG-3'
N307A	Forward: 5'-CTCCCGTAAGTGGGCCCAAAGCACTGCTCGC-3' Reverse: 5'-GCGAGCAGTGCTTTGGGCCCACTTACGGGAG-3'
H338A	Forward: 5'-CAGCTTGGTGGGCCGGACTGCACCCTGG-3' Reverse: 5'-CCAGGGTGCAGTCCGGCCCACCAAGCTG-3'
H341A	Forward: 5'-GCACGGACTGGCCCCTGGACAGGTTTTCGG-3' Reverse: 5'-CCGAAAACCTGTCCAGGGGCCAGTCCGTGC-3'
D358A	Forward: 5'-GTTATGGTGGAGGCCGCCTACCTGATCCAC-3' Reverse: 5'-GTGGATCAGGTAGGCGGCCTCCACCATAAC-3'
H362A	Forward: 5'-GCCGACTACCTGATCGCCTCCTTCGCTAACGAG-3' Reverse: 5'-CTCGTTAGCGAAGGAGGCGATCAGGTAGTCGGC-3'

Table 4.1 Forward and reverse mutagenesis primers used to make the indicated mutations to alanine within hGOAT.

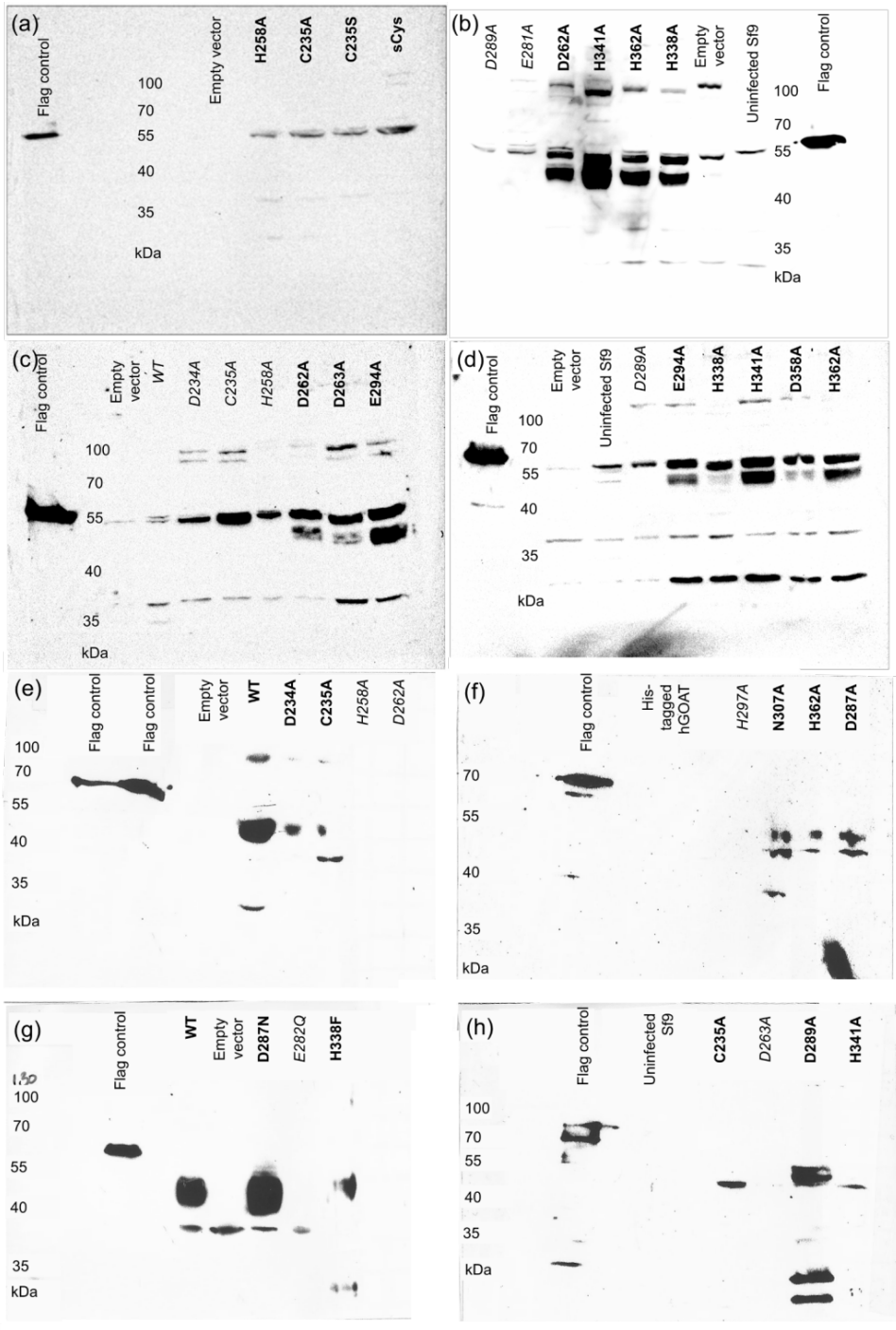
(a)
Flag control
Empty vector
H258A
C235A
C235S
sCys
100
70
55
40
35
kDa
(b)
D289A
E281A
D262A
H341A
H362A
H338A
Empty vector
Uninfected Sf9
Flag control
100
70
55
40
35
kDa
(c)
Flag control
Empty vector
WT
D234A
C235A
H258A
D262A
D263A
E294A
100
70
55
40
35
kDa
(d)
Flag control
Empty vector
Uninfected Sf9
D289A
E294A
H338A
H341A
D358A
H362A
100
70
55
40
35
kDa
(e)
Flag control
Flag control
Empty vector
WT
D234A
C235A
H258A
D262A
100
70
55
40
35
kDa
(f)
Flag control
His-tagged hGOAT
H297A
N307A
H362A
D287A
70
55
40
35
kDa
(g)
Flag control
WT
Empty vector
D287N
E282Q
H338F
100
70
55
40
35
kDa
(h)
Flag control
Uninfected Sf9
C235A
D263A
D289A
H341A
100
70
55
40
35
kDa

Figure 4.2 Expression analysis by Western blot for hGOAT mutant variants containing alanine and conservative mutations, using a C-terminal FLAG epitope tag on all variants. (a) Expression of H258A, C235A, C235S, and sCys supported by a band at ~55 kDa. No bands were present for empty vector microsomal fraction. (b) Expression of D262A, H341A, H362A, and H338A supported by the presence of a band at ~55 kDa. Nonspecific bands just above 55 kDa were present in all samples, including empty vector and uninfected microsomal fraction. (c) Expression of D262A, D263A, and E294A supported by presence of a band at ~55 kDa. Nonspecific bands just above 55 kDa were present in all samples, including empty vector microsomal fraction. (d) Expression of E294A, H338A, H341A, D358A, and H362A supported by the presence of a band at ~55 kDa. Nonspecific bands were present in all samples, including empty vector and uninfected microsomal fraction. (e) Expression of D234A and C235A supported by the presence of a band at ~55 kDa. No bands were present for empty vector microsomal fraction. (f) Expression of N307A and D287A supported by the presence of a band at ~55 kDa. No bands were seen for a His-tagged hGOAT variant, which does not contain the FLAG epitope. (g) Expression of H338F supported by the presence of a band at ~55 kDa. Nonspecific bands were seen in all samples, including empty vector microsomal fraction. (h) Expression of D289A and H341A supported by the presence of a band ~55 kDa. No bands were seen in uninfected microsomal fraction.

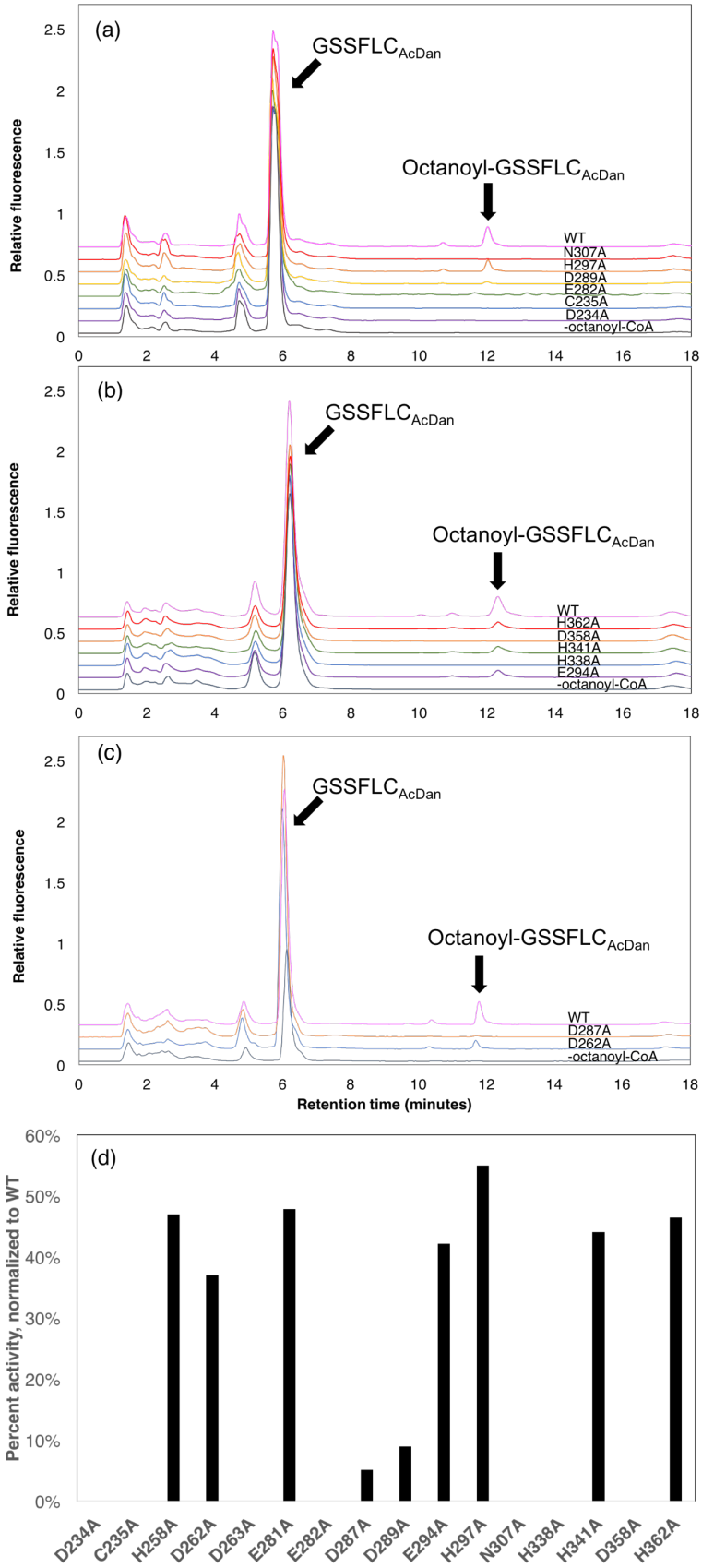
(a)
GSSFLC$_{AcDan}$
Octanoyl-GSSFLC$_{AcDan}$
WT
N307A
H297A
D289A
E282A
C235A
D234A
-octanoyl-CoA
Relative fluorescence
(b)
GSSFLC$_{AcDan}$
Octanoyl-GSSFLC$_{AcDan}$
WT
H362A
D358A
H341A
H338A
E294A
-octanoyl-CoA
Relative fluorescence
(c)
GSSFLC$_{AcDan}$
Octanoyl-GSSFLC$_{AcDan}$
WT
D287A
D262A
-octanoyl-CoA
Relative fluorescence
Retention time (minutes)
(d)
Percent activity, normalized to WT
D234A
C235A
H258A
D262A
D263A
E281A
E282A
D287A
D289A
E294A
H297A
N307A
H338A
H341A
D358A
H362A

Figure 4.3 Activity screening of hGOAT alanine mutations probing for potential general bases. (a) Chromatograms showing $GSSFLC_{AcDan}$ reaction with WT and mutant hGOAT variants, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; N307A, red; H297A, orange; D289A, yellow; E282A, green; C235A, blue; D234A, purple; negative control with no acyl donor, gray. (b) Chromatograms showing $GSSFLC_{AcDan}$ reaction with WT and mutant hGOAT variants, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; H362, red; D358A, orange; H341, green; H338A, blue; E294A, purple; negative control, gray. (c) Chromatograms showing $GSSFLC_{AcDan}$ reaction with WT and mutant hGOAT variants, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; D287A, orange; D262A, blue; negative control, gray. Sets of chromatograms are grouped by reactions performed in parallel. (d) Summary of activity of each hGOAT alanine mutant variant, normalized to the WT enzyme activity run side-by-side.

For positions which resulted in a complete loss of activity when mutated to alanine, primers were designed for a conservative mutation (Table 4.2). Aspartates (D) were mutated to asparagine (N), glutamates (E) to glutamines (Q), histidines (H) to phenylalanine (F), and cysteine (C) to serine (S). These mutations maintain size, structure, and polarity of the natural side chains, but still remove general base capabilities. As for the alanine mutations, expression of conservative hGOAT mutants was supported by Western blot for the C235S and H338F mutants (Figure 4.2a, g). Ghrelin acylation activity was observed with the E282Q mutant, indicating that this residue is not required for ghrelin acylation, but no evidence for octanoylation activity was observed for any of the other conservative hGOAT mutants (Figure 4.4).

Mutations targeting cysteine residues

Based on the susceptibility of hGOAT to cysteine-modifying inhibitors such as the CDDO derivatives and NEM (Chapter 3),[16] we hypothesized that a cysteine residue within the enzyme may be involved in the ghrelin acylation mechanism. Mutation of C235 to either alanine or serine leads to a complete loss of hGOAT acylation activity. To assess whether this is the only functionally required cysteine, we designed a single cysteine hGOAT mutant variant (hGOAT_sCys), in which fifteen of the sixteen cysteine residues within hGOAT were mutated to alanine leaving only C235 intact. Expression of this hGOAT mutant was verified by Western blot against the FLAG epitope tag (Figure 4.2a), but no activity was observed (Figure 4.5a).

D234N	Forward: 5'-GGAGCCGGACTGACTAATTGCCAGCAATTCGAATGT-3' Reverse: 5'-ACATTCGAATTGCTGGCAATTAGTCAGTCCGGCTCC-3'
C235S	Forward: 5'-GCCGGACTGACTGATAGCCAGCAATTCGAATGT-3' Reverse: 5'-ACATTCGAATTGCTGGCTATCAGTCAGTCCGGC-3'
D263N	Forward: 5'-CTGGATCCTCGACAATTCGCTCTTGCACGCTG-3' Reverse: 5'-CAGCGTGCAAGAGCGAATTGTCGAGGATCCAG-3'
E282Q	Forward: 5'-CAGTCCCAGGAGAGCAAGGTTACGTTCCTGAC-3' Reverse: 5'-GTCAGGAACGTAACCTTGCTCTCCTGGGACTG-3'
H338F	Forward: 5'-CTTTCTCAGCTTGGTGGTTCGGACTGCACCCTGGA-3' Reverse: 5'-TCCAGGGTGCAGTCCGAACCACCAAGCTGAGAAAG-3'
D358N	Forward: 5'-GTTATGGTGGAGGCCAACTACCTGATCCA-3' Reverse: 5'-TGGATCAGGTAGTTGGCCTCCACCATAAC-3'

Table 4.2 Forward and reverse mutagenesis primers used to make the indicated mutations to structurally conserved residues within hGOAT.

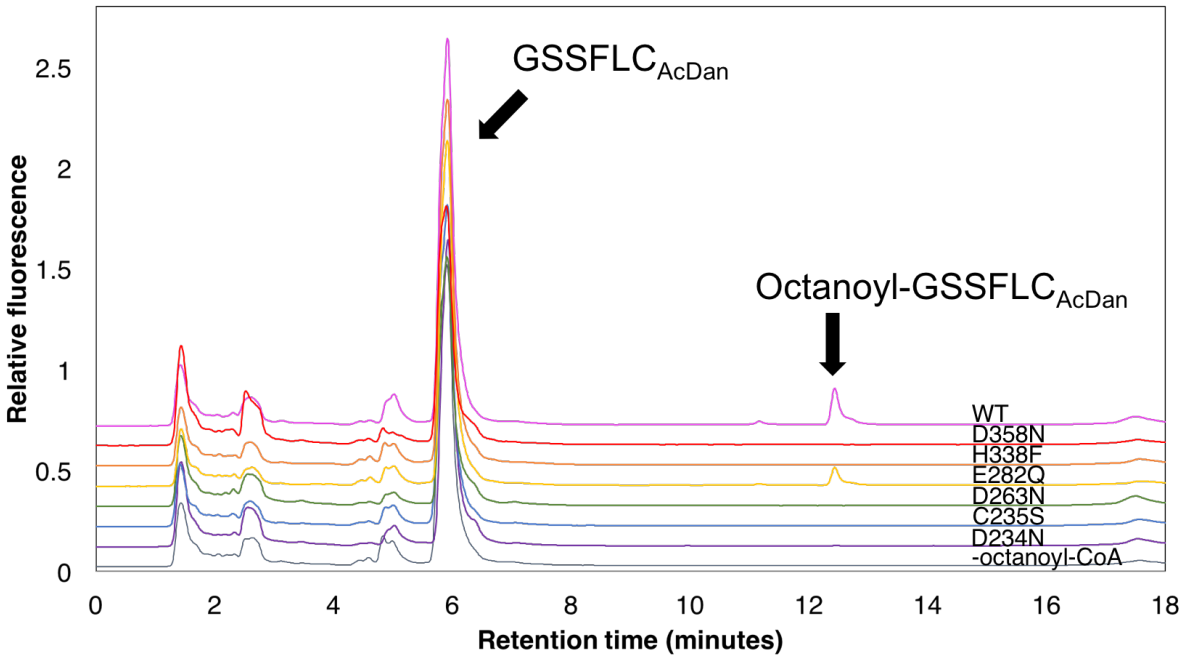

Figure 4.4 Activity screening of hGOAT conservative mutations probing for potential general bases. Chromatograms showing GSSFLC$_{AcDan}$ reaction with WT and mutant hGOAT variants, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; D358N, red; H338F, orange; E282Q, yellow; D263N, green; C235S, blue; D234N, purple; negative control with no acyl donor, gray. Reactions were run in parallel.

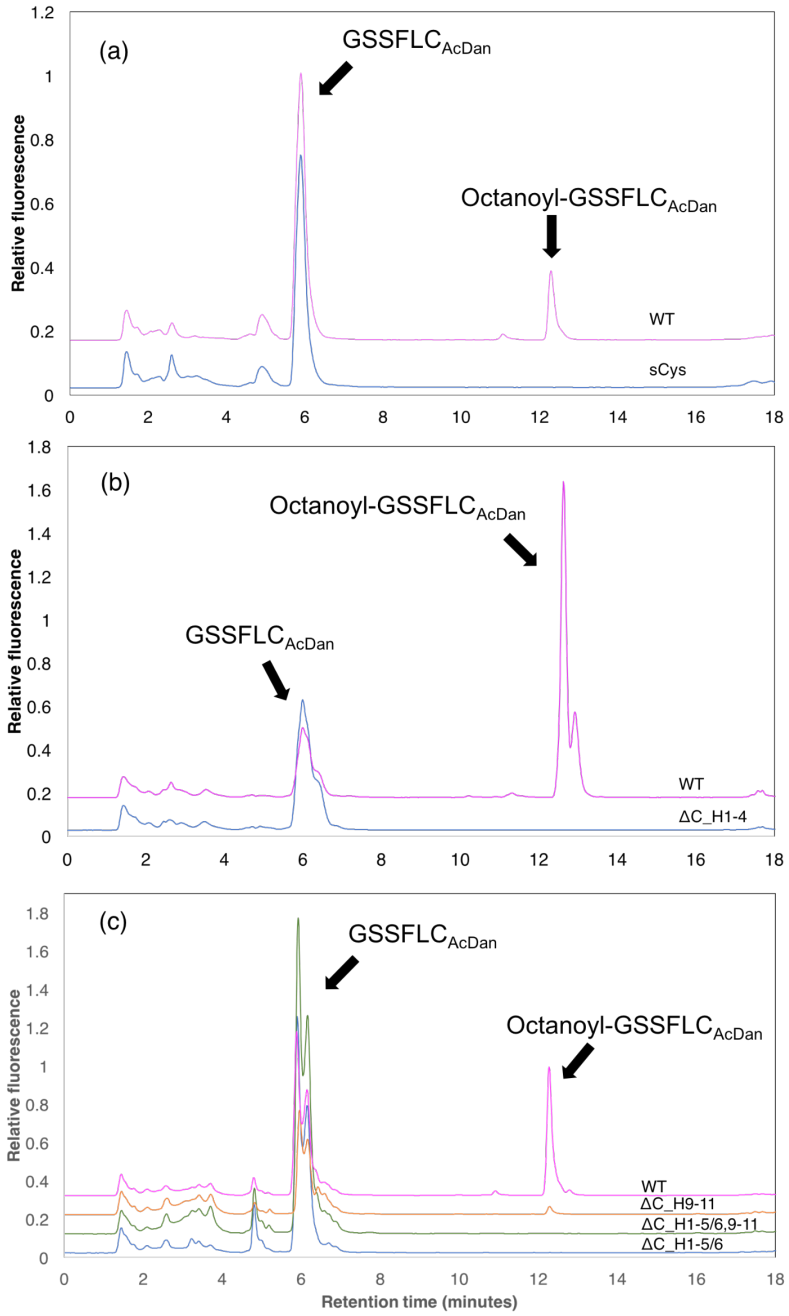

Figure 4.5 Activity screening of hGOAT mutant variants probing the role of cysteines within hGOAT. (a) Chromatograms showing GSSFLC$_{AcDan}$ reaction with WT and sCys hGOAT, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; sCys, blue. (b) Chromatograms showing GSSFLC$_{AcDan}$ reaction with WT and hGOAT_ΔC_H1-4, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; _ΔC_H1-4, blue. (c) Chromatograms showing GSSFLC$_{AcDan}$ reaction with WT and hGOAT_ΔC variants, with peptide substrate eluting at ~6 minutes and octanoylated peptide product eluting at ~12 minutes: WT, magenta; ΔC_H1_9-11, orange; ΔC_H1_1-5/6,9-11, green; ΔC_H1_1-5/6, blue. Sets of chromatograms are grouped by reactions performed in parallel.

Next we designed mutant variants of hGOAT in which all cysteines within enzyme topological subdomains were mutated to serine (Figure 4.6a). All cysteines from the N-terminus to the end of transmembrane (TM) helix 4 were mutated to serine in hGOAT_ΔC_H1-4; all cysteines from the N-terminus to the end of the reentrant loop between TM helices 5 and 6 were mutated in hGOAT_ΔC_H1-5/6; all cysteines within TM helices 9 and 11 were mutated in hGOAT_ΔC_H9-11; and all cysteines except for those within TM helices 6 and 7 were mutated in hGOAT_ΔC_H1-5/6,9-11. Expression of these hGOAT mutants was confirmed by Western blot against a C-terminal FLAG epitope tag (Figure 4.6b, c). Of these four mutant variants, only hGOAT_ΔC_H9-11 showed any observable activity in our *in vitro* enzyme assay indicating that the cysteines within this region (C350, C409, C421, and C434) are not absolutely required for activity (Figure 4.5b, c). Investigation into the role of individual cysteine residues within TM helices 1-8 is an ongoing project. Primers have been designed to mutate each of these cysteines to serines individually, and the activity of the resulting hGOAT mutant variants will reveal the specific requirement of each cysteine residue.

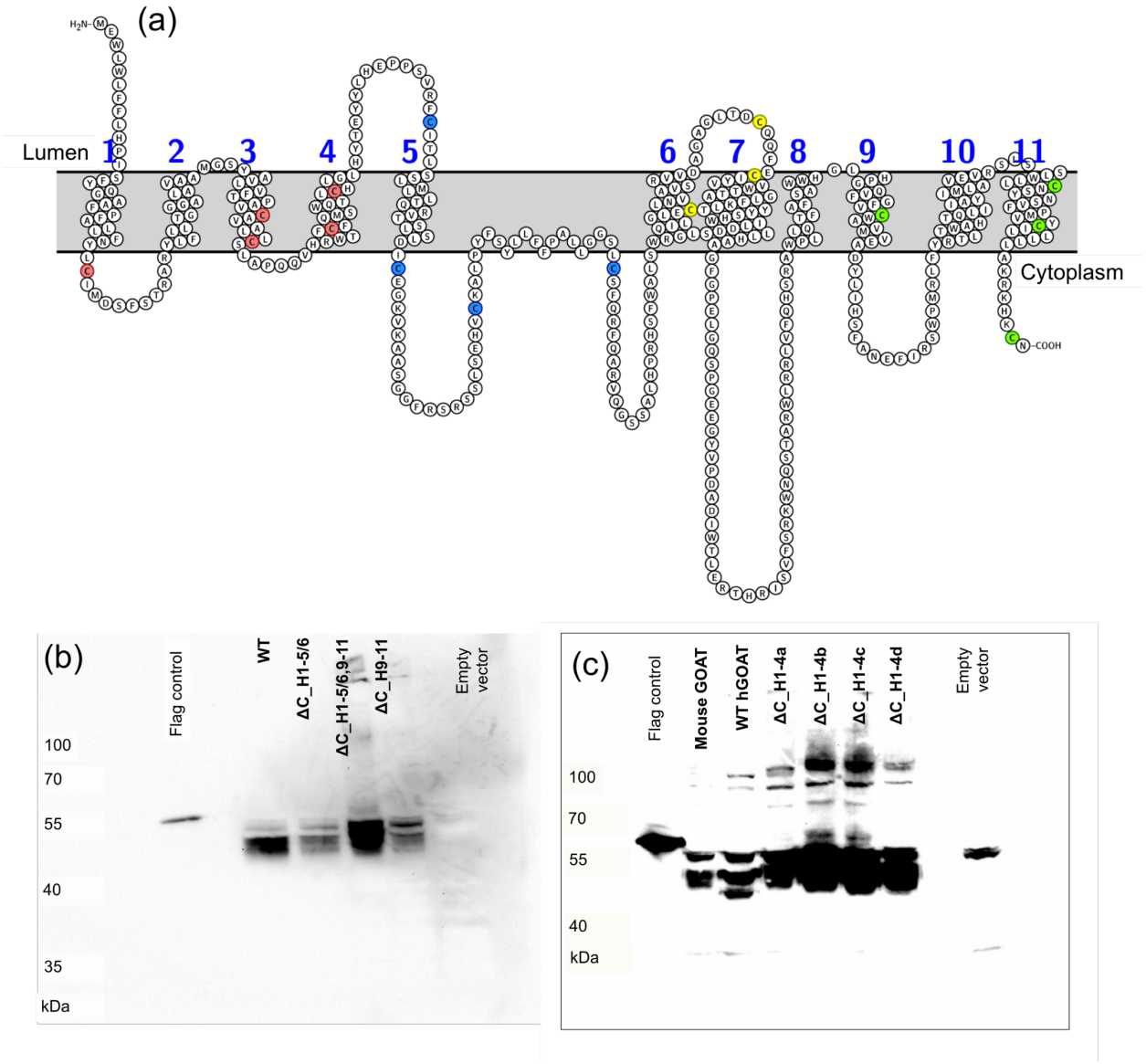

Figure 4.6 Design and expression analysis of hGOAT variants targeting cysteines within topological subdomains. (a) Topological model of hGOAT with cysteines highlighted corresponding to topological subdomains. Cysteines within subdomain spanning TM helices 1 – 4 are highlighted in red; cysteines within subdomain spanning TM helix 5 through the reentrant loop are highlighted in blue; cysteines within subdomain spanning TM helices 6 – 8 are highlighted in yellow; cysteines within subdomain spanning TM helices 9 – 11 are highlighted in green. The model was constructed by comparison to the experimentally developed topology model for mouse GOAT using the Protter online server.[11, 18] (b) Expression of ΔC_H1-5/6, ΔC_H1-5/6,9-11, and ΔC_H9-11 supported by the presence of bands at ~55 kDa. No bands were seen in empty vector microsomal fraction. (c) Expression of several preparations of ΔC_H1-4 supported by the presence of bands at ~55 kDa. Different preparations correspond to different amounts of virus added to optimize expression of the enzyme. Nonspecific bands just above 55 kDa were present in all samples, including empty vector microsomal fraction.

4.3 Discussion and conclusions

Mutagenesis studies described here have revealed a number of residues within the human isoform of GOAT that are required for activity. Among these functionally required residues are N307 and H338, which are conserved across MBOAT family enzymes[3] and proposed to be involved in catalysis, confirming previous findings by other groups' investigations of the mouse and human isoforms of the enzyme.[1, 2] In addition to these conserved residues, we identified four additional residues – D234, C235, D263, and D358 – that are required for hGOAT activity. No activity was observed when these residues were mutated to either alanine or a structurally conservative residue. Expression of the D234N, D263N, and D358N hGOAT mutants could not be confirmed by Western blot, so the lack of activity with these constructs may be due to lack of expression rather than loss of a functionally required residue. The effects of single-point mutations on hGOAT acylation activity are summarized in Figure 4.7.

Mutation of all cysteines in hGOAT except for C235 to alanine results in complete loss of activity, though expression of the enzyme was confirmed, indicating that C235 is not sufficient for catalytic activity. The series of hGOAT mutants targeting cysteines within specific regions of the enzymes demonstrated that at least one of the cysteines within TM helices 1 – 4 is required for activity. Acylation activity is maintained when all four cysteines within TM helices 9 – 11 are maintained, indicating that these residues are not functionally required.

The functional requirement for C235 and at least one other cysteine along with the susceptibility of hGOAT to inhibition by NEM[16] provide the first indication that an MBOAT family member contains a functionally required cysteine. Hhat contains multiple palmitoylated cysteines, but activity of variants with these cysteines mutated to alanine were not determined.[5] PORCN also contains at least one palmitoylated cysteine residue, but mutation of this residue to

alanine resulted in acylation activity similar to that of the WT enzyme.[8] Determining the individual contribution of each cysteine within helices 1 through 4 of hGOAT, as well as cysteines in helices 5 through 8, may illuminate their functional role in catalysis.

These mutagenesis studies constitute the first steps to locating the active site of hGOAT and determining the catalytic mechanism for ghrelin acylation. H338 is a likely candidate for the general base, given its conservation across species[1, 11] and other MBOAT enzymes.[3] Based on the proposed topology of GOAT,[11] H338 may be located near two other functionally required residues, D234 and C235, opening the possibility that multiple residues in this region are involved in catalysis. Ongoing studies, both in our research group and through collaborations, will use these findings and future experiments to determine the structural and mechanistic basis for hGOAT-catalyzed ghrelin acylation.

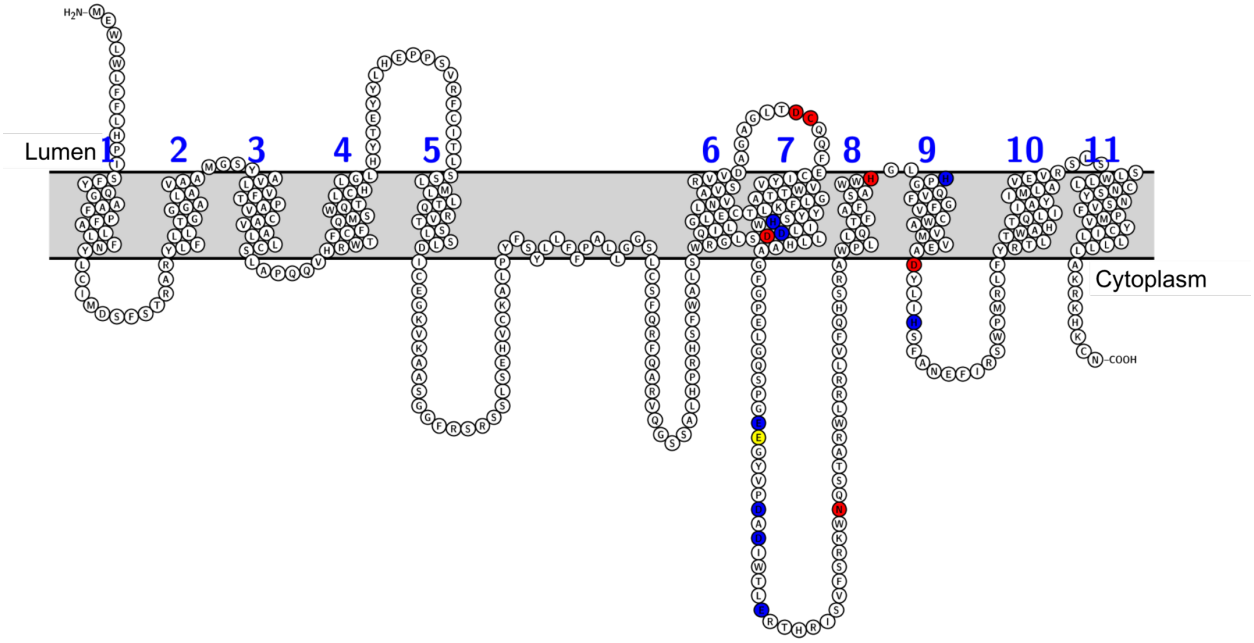

Figure 4.7 Topological model of hGOAT. Residues which resulted in loss of activity when mutated to either alanine or a structurally conservative residue are colored in red. Residues which retained activity when mutated to alanine are colored in blue. E282, which either did not express or resulted in loss of activity when mutated to alanine, but maintained activity when mutated to glutamine, is colored in yellow. The model was constructed by comparison to the experimentally developed topology model for mouse GOAT using the Protter online server.[11, 18]

4.4 Materials and methods

General

Mutagenesis primers were synthesized by Integrated DNA Technologies (Coralville, IA), and were dissolved in purified water prior to use, with concentrations determined by UV absorbance at 260 nm. Genes for hGOAT_sCys, hGOAT_ΔC_H1-4, hGOAT_ΔC_H1-5/6, hGOAT_ΔC_H1-5/6,9-11, and hGOAT_ΔC_H9-11 were synthesized Bio Basic Inc. (Markham, ON, Canada) in the pUC57 vector and were dissolved in purified water prior to use. Methoxy arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical (Ann Arbor, MI) as a stock in methyl acetate and diluted in DMSO prior to use. Octanoyl coenzyme A (octanoyl CoA) was purchased from Advent Bio (Downers Grove, IL) solubilized to 5 mM in 10 mM Tris-HCl (pH 7.0), aliquoted into low-adhesion microcentrifuge tubes, and stored at -80 °C. Acrylodan (Anaspec) was solubilized in acetonitrile, with the stock concentration determined by absorbance at 393 nm upon dilution into methanol (ε = 18,483 M^{-1} cm^{-1} per manufacturer's data sheet). The $GSSFLC_{NH2}$ peptide for fluorescent labeling with acrylodan was synthesized by Sigma-Genosys in the Pep-screen format, and solubilized in 1:1 acetonitrile:H_2O and stored at -80 °C. Peptide concentration was determined by absorbance at 412 nm following reaction of the cysteine thiol with 5,5'-dithiobis(2-nitrobenzoic acid) using ε = 14,150 M^{-1} cm^{-1}.[19]

Single site mutagenesis of hGOAT plasmid DNA

PCR mutagenesis reactions (50 μl total volume) contained 1x Pfu reaction buffer, 10 ng pFastBacDual_MBOAT4i_HA/FLAG/His_6 template DNA previously prepared in our lab, 125 ng forward primer, 125 ng reverse primer, 20 μM dNTPs, and 1 μL (2.5 units) Pfu Turbo DNA polymerase (Agilent). The thermocycler program for PCR mutagenesis proceeded as follows:

initial denaturation (94 °C, 5 min); 30 cycles of denaturation (94 °C, 30 s), annealing (56 °C, 1 min), and extension (68 °C, 2 min); final extension (68 °C, 5 min). 1 μL (20 units) of *Dpn*I was added and reactions were incubated at 37 °C for 1 hr. After *Dpn*1 digestion, 5 μL of the PCR reaction mixture was transformed into 50 μL of chemically competent DH5α cells (Zymo Research) followed by incubation on ice for 30 min. Transformed bacteria were spread on LB-ampicillin (100 μg/mL) plates and incubated at 37 °C overnight. Single colonies were then inoculated into 5 mL LB media containing 100 μg/mL ampicillin in sterile culture tubes, which were incubated at 37 °C overnight with shaking (225 rpm). Following overnight growth, plasmids were purified from cultures using EZ-10 Spin Column Plasmid DNA kit (Bio Basic) per manufacturer's instructions. Single site mutations were verified by DNA sequencing (Genewiz).

Construction of sCys hGOAT plasmid DNA

200 μg of DNA in pUC57 vector was transformed into 50 μL DH5α *E. coli* cells, and incubated on ice for 20 min. The cells were then spread onto LB-ampicillin (100 μg/mL) plates and incubated at 37 °C overnight. A single transformed colony was plucked and inoculated into a 5 mL LB culture containing 100 μg/mL ampicillin, and grown overnight at 37 °C with shaking (225 rpm). Following growth, plasmids were purified from the culture using EZ-10 Spin Column Plasmid DNA kit (Bio Basic) per manufacturer's instructions. Plasmids were stored at -20 °C.

Genes were ligated into pFBD-hGOAT-HA/Flag/His_6 DNA vector using *Eco*RI and *Xho*I restriction sites. 3 μg pFBD-hGOAT-HA/Flag/His_6 DNA was digested with 1 μL (20 units) *Eco*RI, 1 μL (20 units) *Xho*I, 2 μL buffer 4 (20 μL total volume), and 1 μg pUC57 mutant hGOAT DNA was digested with 0.5 μL (10 units) *Eco*RI, 0.5 μL (10 units) *Xho*I, 2 μL buffer 4 (20 μL total volume), and incubated at 37 °C for 2 h. Samples were then run on a 0.8% agarose gel at 110 V

for ~1 h. The bands for the pFBD vector and the mutant hGOAT insert were each cut out and DNA was purified using EZ-10 Gel Extraction kit (Bio Basic) per manufacturer's instructions, and concentrations were measured by UV absorbance at 260 nm.

Ligations were performed with 25 ng of vector DNA, 17 ng insert DNA, 10 μL quick ligase buffer, and 1 μL Quick Ligase (total volume 10 μL) and incubated on ice for 5 min. 5 μL of ligation reaction was added to 50 μL DH5α cells, followed by 30 min incubation on ice. Cells were spread on LB-ampicillin (100 μg/mL) plates and incubated at 37 °C overnight. Single colonies were inoculated into 5 mL LB cultures containing 100 μg/mL ampicillin and grown overnight at 37 °C with shaking (225 rpm). Following overnight growth, plasmids were purified from cultures using EZ-10 Spin Column Plasmid DNA kit (Bio Basic) per manufacturer's instructions, and successful ligation was confirmed by DNA sequencing (Genewiz).

Expression and enrichment of hGOAT mutant variants

Mutant hGOAT genes within the pFastBacDual vector were transformed into DH10Bac *E. coli* cells to produce bacmid encoding the mutant hGOAT using the Bac-to-Bac expression system (Invitrogen). Sf9 insect cells (5.0×10^8 cells in a 500 mL total culture volume) were infected with hGOAT baculovirus at a multiplicity of infection of 10 followed by protein expression for 40 h at 28 °C shaking 150 rpm. Cells were harvested by centrifugation (500 x *g*, 5 min), and freezing the pellet at -80 °C. The cell pellets were thawed on ice, resuspended in 25 mL lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.0), 1 mM sodium ethylenediamine tetraacetate (NaEDTA), 1 mM dithiothreitol (DTT), complete protease inhibitor (Roche), 10 μg/mL pepstatin A, and 100 μM bis(4-nitrophenyl)phosphate]. The resuspended cells were lysed with a Dounce homogenizer on ice, followed by removal of cell debris by centrifugation (3,000 x *g*, 4 °C, 10 min). The microsomal

fraction was then isolated by ultracentrifugation of the supernatant (100,000 x *g*, 4 °C, 1 hr). The isolated microsomal fraction pellet was resuspended in 50 mM HEPES (pH 7.0) and stored in low-adhesion microcentrifuge tubes at -80 °C until use.

Peptide substrate fluorescent labeling

$GSSFLC_{NH2}$ (300 μM) and acrylodan (500 μM) were dissolved in 500 μL 1:1 50 mM HEPES (pH 7.8):acetonitrile, followed by incubation at room temperature in the dark for 18 hr with shaking. Acrylodan-labeled peptide was purified by reverse phase HPLC (Zorbax Eclipse XDB column, 9.4 x 250 mm) using an isocratic mobile phase of water containing 0.05% trifluoroacetic acid (TFA) (65%) and acetonitrile (35%) flowing at 4.2 mL/min over 21 min). Labeled peptide eluted around 8 min, monitoring UV absorbance at 360 nm. Collected fractions containing labeled peptides were dried under vacuum at room temperature and resuspended in 1:1 acetonitrile:H_2O, and labeling was confirmed by MALDI-TOF mass spectrometry (Bruker Autoflex III, SUNY-ESF) using a matrix of saturated sinapinic acid in 0.1% TFA and 50 mM ammonium phosphate. Concentration of acrylodanylated peptide was determined by absorbance of acrylodan at 360 nm (ε = 13,300 M^{-1} cm^{-1}), and was stored at -80 °C.

hGOAT activity assays and analysis

Microsomal fraction form Sf9 cells expressing WT or mutant hGOAT were thawed on ice and passed through an 18-gauge needle 10 times to homogenize the fraction. Assays were performed with ~100 μg of membrane protein, as determined by a Bradford assay. The microsomal fraction was preincubated with 1 μM MAFP in 50 mM HEPES (pH 7.0) for 30 minutes at room temperature.[20] Reactions were initiated with the addition of 500 μM octanoyl CoA and 1.5 μM

fluorescently labeled ghrelin mimetic, $GSSFLC_{AcDan}$, in a total volume of 50 μL, incubated for 3 hr at room temperature, and stopped with the addition of 50 μL of 20% acetic acid in isopropanol, and solutions were clarified by protein precipitation with 16.7 μL of 20% trichloroacetic acid (TCA) followed by centrifugation (1,000 x *g,* ~1 min). The supernatant was analyzed by reverse phase HPLC.

Assay samples were analyzed on an Agilent 1260 HPLC with a C18 reverse phase column (Zorbax Eclipse, 4.6 x 150 mm) using a gradient of 30% acetonitrile in 0.05% TFA to 63% acetonitrile in 0.05% TFA over 14 min, followed by 100% acetonitrile for 10 min. Fluorescent peptides were detected by fluorescence (λ_{ex} = 360 nm, λ_{em} = 485 nm), with the unacylated peptide eluting with a retention time of ~6 min and the octanoylated peptide product eluting with a retention time of ~12 min. Chromatogram analysis and peak integration was performed using Chemstation for LC (Agilent Technologies).

Confirmation of expression by Western blot

Microsomal fractions were thawed on ice and passed through an 18-guage needle ten times to homogenize. Membrane protein concentration was determined by Bradford assay, and samples for gel analysis were prepared to contain 50 μg protein, 1x sample buffer (0.33 M Tris HCl pH 6.8, 0.1 M SDS, 14% glycerol, and 0.5 M DTT), and 50 mM HEPES pH 7.0 in a total volume of 45 μL. Samples were heated at 50.2 °C for 5 min, followed by incubation at room temperature for 15 min. Samples were loaded onto a 10% SDS-polyacrylamide protein gel, and gels were run at 120 V for 1.5 hr. Proteins were then transferred to a polyvinylidene fluoride membrane using a Bio Rad Trans-Blot Turbo Transfer System at 1.3 A / 25 V for 30 min.

The membrane was blocked with 5% nonfat milk for 3 hr at room temperature with gentle rocking, and then incubated with the Flag antibody, OctA-probe-HRP rabbit polyclonal antibody (Santa Cruz Biotechnologies), in 5% nonfat milk (1:200 dilution, 10 mL total volume) overnight at 4 °C with gentle rocking. Proteins bearing the Flag epitope were detected using a chemiluminescent substrate (Thermo Fischer Scientific) and imaged. hGOAT and hGOAT mutant variants are detected by the presence of a band at ~55 kDa.

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Chapter 5: Development of a fluorescence-based high-throughput assay for rapid identification of GOAT inhibitors

5.1 Introduction

As described in Chapter 1, GOAT is an attractive target for the therapeutic modulation of ghrelin signaling. Ghrelin is the only predicted substrate for GOAT within the human proteome,[1-3] and its octanoylation is absolutely necessary for its binding and activation of GHSR1a.[1] Although antagonists and agonists of the GHSR1a receptor have been extensively studied,[4, 5] and despite the potential for GOAT inhibitors as a novel avenue for controlling ghrelin signaling, there are only a small number of reported GOAT inhibitors.[6-10] Only one GOAT inhibitor – GO-CoA-Tat – has been reported to be effective in cell and animal studies.[7, 11, 12]

The lack of reported GOAT inhibitors is due in part to experimental limitations of GOAT activity assays. Reported GOAT activity assays employing recombinantly expressed enzyme in microsomal fraction have determined GOAT activity by the detection of radiolabeled octanoate incorporation,[6, 7, 13, 14] detection of acylated ghrelin by radiolabeled anti-ghrelin antiserum,[15] modification and detection of octynoate incorporation,[16] or detection of increased peptide hydrophobicity by reverse phase HPLC.[17] Each of these assays requires extensive work-up after reaction completion, making the analysis of large quantities of reactions difficult and time-consuming. There are currently no cell-based screening assays reported for identifying GOAT inhibitors, unlike those established for its MBOAT relative PORCN.[18]

In our efforts to identify novel small-molecule inhibitors of hGOAT, we have utilized our HPLC-based assay to screen a library of diverse small molecules from the NIH Developmental Therapeutics Program. Through this screen, we successfully identified a class of synthetic triterpenoids which covalently and reversibly inhibit hGOAT in the low micromolar range.[10]

However, this screen has been relatively low-throughput, with only 478 compounds out of the 1,596-compound library screened since its acquisition by the Hougland laboratory in 2014. Clearly, more efficient identification of potent GOAT inhibitors requires an assay amenable to high-throughput screening.

An alternative method to potentially detect acylation of ghrelin involves the fluorescently-labeled ghrelin peptide substrates already used in our lab. Acrylodan, an environmentally-sensitive fluorophore, increases in fluorescence when in a hydrophobic environment.[19-21] Octanoylation of a peptide labeled with acrylodan should result in an increase of peptide fluorescence within the reaction mixture, which could be detected without the need to separate reaction products. Modification of the fluorescent peptide GOAT substrate previously reported by our research group[17] has enabled sufficient fluorescence enhancement of the octanoylated peptide to monitor GOAT activity without separation by HPLC. Without the need for separation of reaction components, this modified assay could be adapted to a high-throughput format using a 96-well plate fluorescent plate reader. As a proof of principle, the NIH Clinical Collection (NCC) library of small molecules was screened using this assay.

5.2 Results

Development of a modified fluorescent ghrelin substrate

Previous attempts to monitor GOAT octanoylation activity by an increase in fluorescence of the solvatochromic acrylodan fluorophore on the ghrelin mimetic substrate $GSSFLC_{AcDan}$ were unsuccessful (K. McGovern-Gooch and J. Darling, unpublished data). Addition of the fluorescently labeled $GSSFLC_{AcDan}$ substrate into microsomal fraction resulted in an increase in fluorescence independent of the presence of GOAT or the addition of the octanoyl CoA acyl donor. As this fluorescence enhancement was not dependent on GOAT activity, a likely explanation is the acrylodan fluorescence increases due to partitioning of the fluorescent substrate into the hydrophobic microsomal fraction.

One avenue for increasing the fluorescence signal upon peptide octanoylation would be modification of the ghrelin substrate peptide to generate greater fluorescence enhancement upon acylation. Improved expression conditions which lead to increased hGOAT activity and improved product stability with the incorporation of MAFP[22] led us to revisit exploration of a fluorescence-based assay with a novel peptide substrate.

Our standard ghrelin peptide substrate, $GSSFLC_{AcDan}$, exhibits a modest increase in fluorescence upon acylation of serine 3.[17] In this peptide, the acylation site at S3 lies three amino acids away from the acrylodan fluorophore. If possible, moving the fluorophore position closer to the acylation site could lead to an increased fluorescence change due to proximity to the hydrophobic octanoyl group. Incorporation of the acrylodan at serine 2 ($GC_{AcDan}SFLS$) led to an inactive substrate, with subsequent studies indicating a steric constraint at the S2 position.[3, 17] However, hGOAT is tolerant to mutations of F4, modifying substrates with a wide range of amino acids at this position, including those with large side chains such as tryptophan.[3] Mutation of F4

to a cysteine allows the incorporation of acrylodan at the residue directly adjacent to the acylation site, leading to an even greater degree of fluorescence enhancement. This peptide, GSSCLS, was successfully labeled with acrylodan as confirmed by MALDI-TOF (Figure 5.1a), and was octanoylated when incubated with octanoyl CoA and hGOAT in our standard HPLC-based assay (Figure 5.1b).

Development of a real-time fluorescence-based hGOAT activity assay

We next sought to determine whether acylation of $GSSC_{AcDan}LS$ substrate could be detected by an increase in fluorescence on a fluorescent plate reader, and if so, determine the optimal conditions for reaction monitoring. At a microsomal fraction concentration of 10% v/v (10 μL in a 100 μL reaction volume, corresponding to ~50 μg protein) and $GSSC_{AcDan}LS$ concentration of 0.5 μM, an increase in fluorescence over a three-hour reaction time was seen in the presence, but not the absence, of 500 μM octanoyl CoA (Figure 5.2). At higher concentrations of peptide, nonspecific fluorescence enhancement was observed in the absence of octanoyl CoA, consistent with fractionation of the fluorescent peptide into the lipid microsomes.

To confirm that the increase in fluorescence at low peptide concentrations was specific to the acylation of the ghrelin substrate, we performed controls in which we included empty vector microsomal fraction (which does not contain hGOAT) or a GOAT inhibitor [Dap^3]octanoyl-ghrelin(1-5)-NH_2 (10 μM, IC_{50} ~30 nM) in the presence of 500 μM octanoyl CoA. In these negative control reactions, there was no increase in fluorescence over the time course of observation, similar to reactions performed in the absence of octanoyl CoA (Figure 5.3).

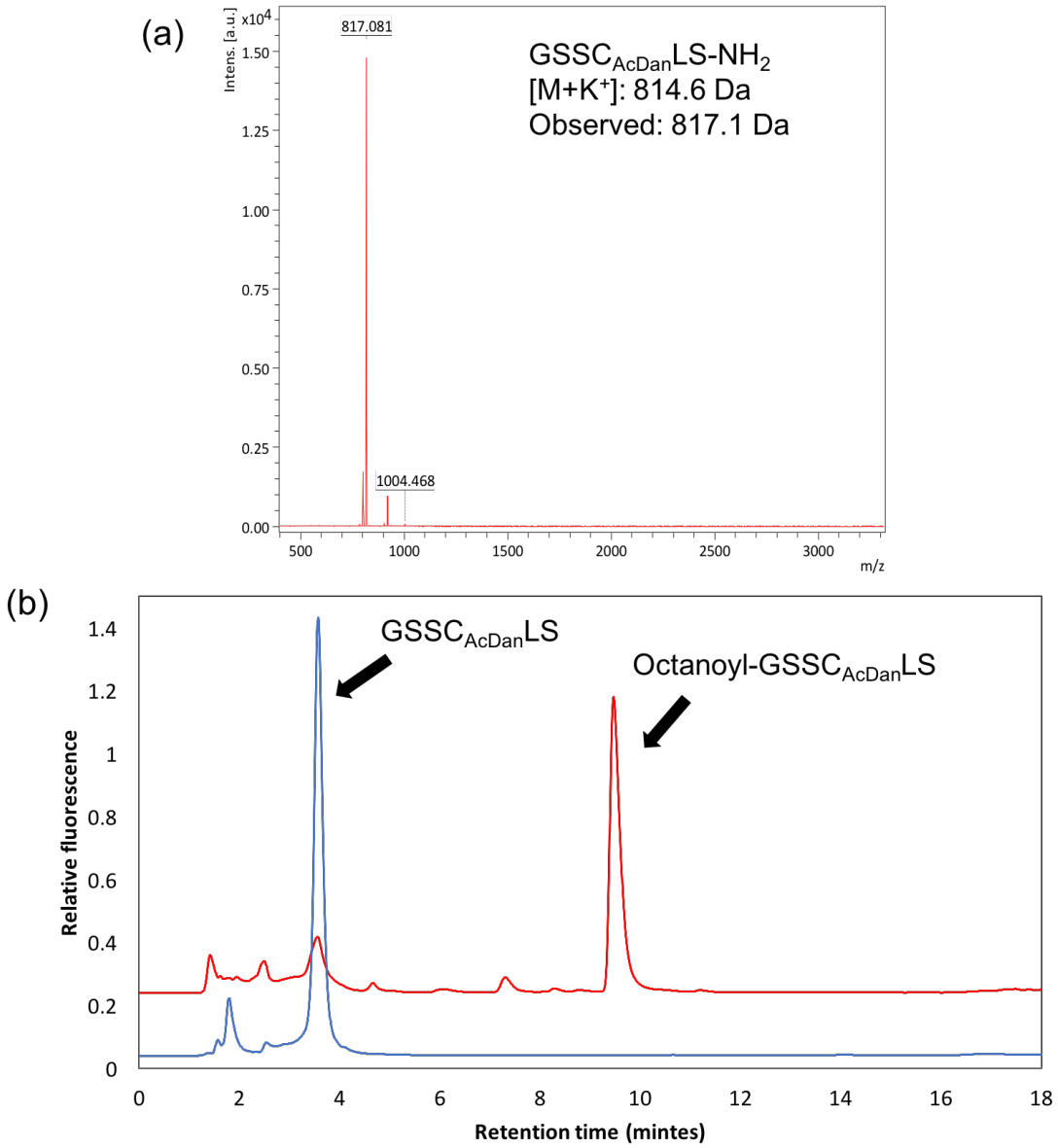

Figure 5.1 Modified ghrelin peptide GSSC$_{AcDan}$LS is a substrate of hGOAT. (a) MALDI-TOF confirmation of acrylodan labeling of cysteine residue of GSSCLS$_{NH2}$. Expected mass of potassium adduct = 814.6 Da, observed mass = 817.1 Da. An external standard with an expected mass of 1089.5 Da for the potassium adduct also displayed a mass difference of +2 Da, indicating successful labeling of GSSC$_{AcDan}$LS. (b) HPLC chromatogram of GSSC$_{AcDan}$LS in reaction buffer (blue trace) and reaction of GSSC$_{AcDan}$LS with hGOAT and octanoyl CoA (red). The unacylated peptide elutes with a retention time of ~4 minutes, and the octanoylated product elutes with a retention time of ~10 minutes.

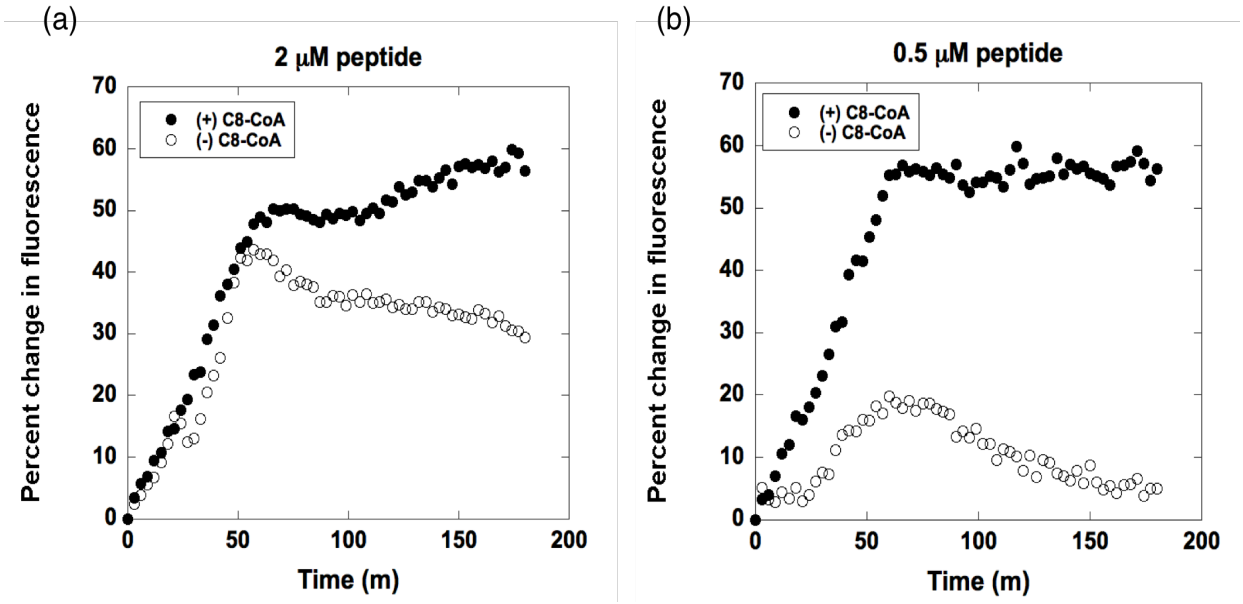

Figure 5.2 Low peptide concentrations minimize non-specific fluorescence enhancement. (a) Fluorescence of 2 μM $GSSC_{AcDan}LS$ increases to a similar degree in the presence (closed circles) and absence (open circles) of octanoyl CoA over time when incubated with hGOAT microsomal fraction, indicating that fluorescence enhancement is not specific to acylation of peptide under these conditions. (b) Fluorescence of 0.5 μM $GSSC_{AcDan}LS$ increases in the presence of octanoyl CoA (closed circles), but to a much lesser degree in the absence of octanoyl CoA (open circles) when incubated with hGOAT microsomal fraction. Fluorescence enhancement is given as the percent increase in fluorescence relative to fluorescence at $time_0$, as described in the Methods section.

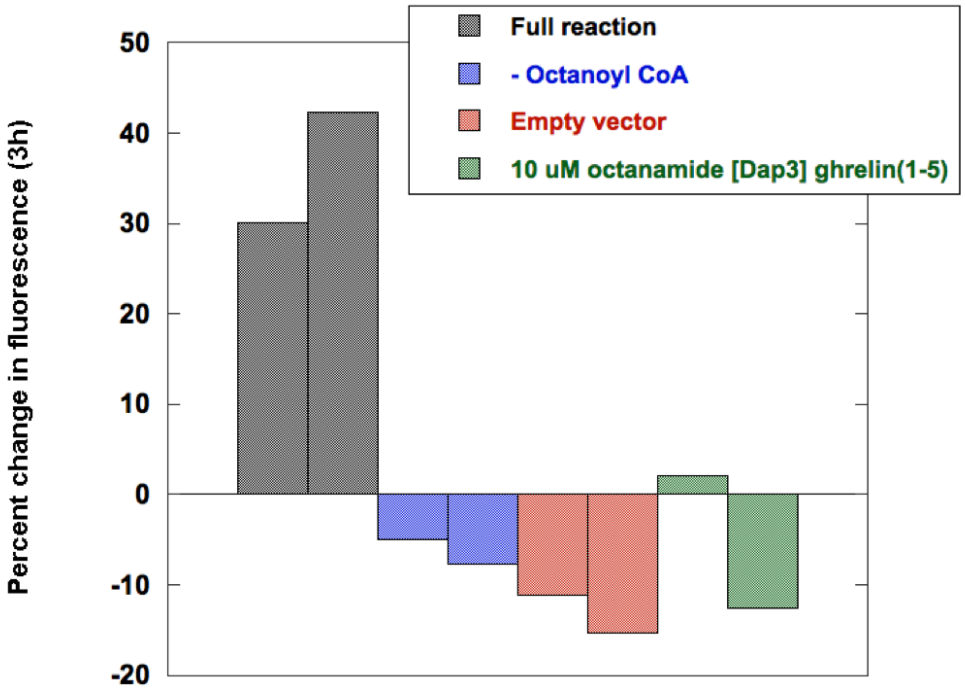

Figure 5.3 Fluorescence enhancement is specific to acylation of $GSSC_{AcDan}LS$ peptide. The fluorescence of the peptide increases over the three-hour reaction time relative to the fluorescence at $time_0$ in the complete reaction (black bars). In the absence of octanoyl CoA (blue bars), in the absence of hGOAT (red bars), or in the presence of a high concentration of GOAT inhibitor $[Dap^3]$octanoyl-ghrelin(1-5)NH_2^6 (green bars), the fluorescence either decreases over the course of the reaction or minimally increases. Reactions were performed in duplicate, and change in fluorescence is given as the percent difference in fluorescence at three hours relative to fluorescence at $time_0$ as described in the Methods section.

Application of real-time fluorescence-based hGOAT activity assay to screen the NCC library

As the fluorescence assay employing the $GSSC_{AcDan}LS$ substrate proved amenable to a plate-based high-throughput format, we applied this assay to perform a screen of the NIH Clinical Collection (NCC) library of small molecule compounds for hGOAT inhibitors. For initial screening, each compound in the NCC was tested at 100 μM for inhibition of hGOAT activity. As a positive inhibitor control, the CDDO-EA small molecule hGOAT inhibitor previously identified by our laboratory with an IC_{50} of 8 ± 4 μM was included in the screen.[10] CDDO-EA inhibits hGOAT activity to a degree where no fluorescence increase is seen in this assay when present at 100 μM, similar to a negative control with octanoyl CoA absent from the reaction. For each of the 700 compounds of the NCC library, the percent change in fluorescence at 3 hours and the fold inhibition was calculated. Compounds resulting in an overall reduction in fluorescence or a calculated fold inhibition greater than 10 were assigned a fold inhibition of 10 as a lower limit. Across the NCC library, the average fold inhibition was 2.6 ± 2.9 with 10.4% of the compounds exhibiting fold inhibitions more than 2.5 standard deviations from the mean, all but one of which had met the 10-fold inhibition upper limit (Figure 5.4). To complete investigation of this library of compounds, they will be screened at lower concentrations. Those that inhibit hGOAT at lower concentrations will be validated by inhibition against hGOAT in the HPLC-based activity assay, which provides a precise measurement of both acyl peptide product and unacylated peptide substrate.

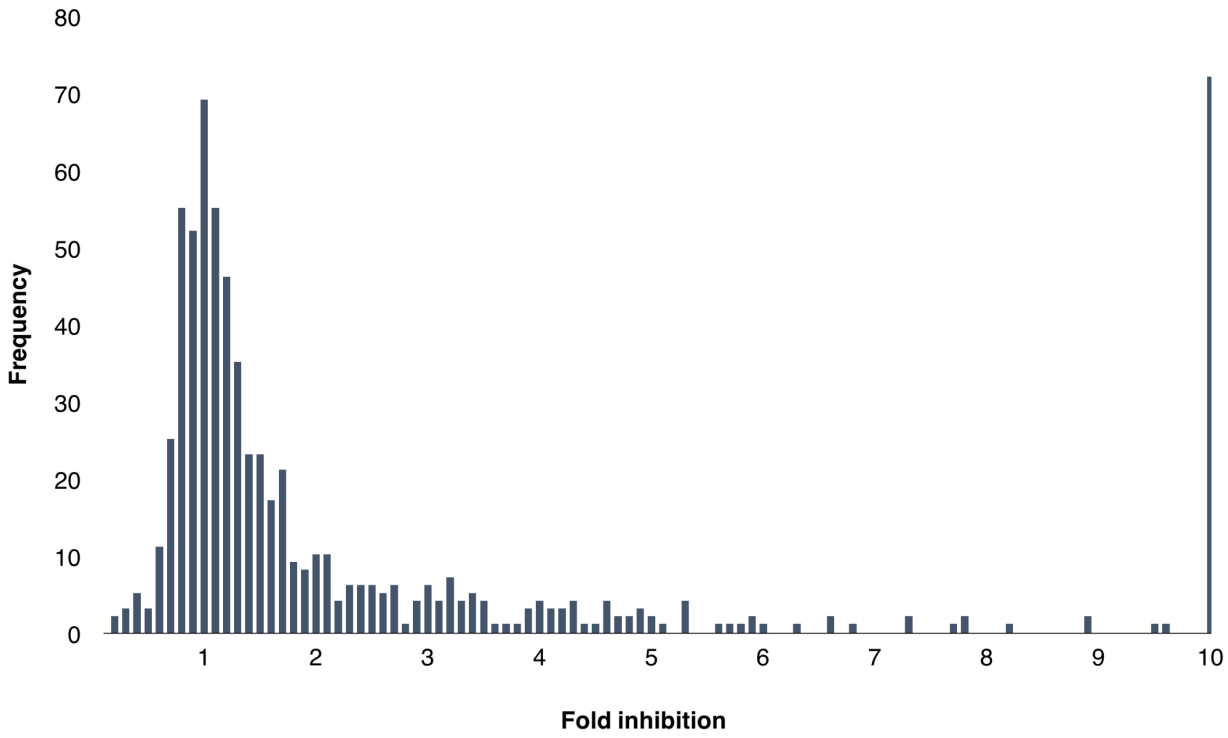

Figure 5.4 NCC library screening for hGOAT inhibitory activity at 100 μM. Screening results are presented as a histogram of the number of compounds exhibiting a specific fold inhibition, with fold inhibition defined as the percent change in fluorescence of the vehicle reaction divided by the percent change in fluorescence of the reaction containing a test compound. Ten-fold inhibition was set as the upper limit, and results were binned by every 0.1% inhibition.

5.3 Discussion and conclusions

Since the discovery of GOAT, biochemical investigations of ghrelin octanoylation by this enzyme have been limited by the lack of versatile, robust assays. All reported *in vitro* GOAT activity assays have required extensive workup following reaction completion[6, 7, 13-17] limiting their application to high-throughput formats. While Garner and Janda's cat-ELCCA assay[16] can be performed in a 96-well plate and was used to screen for inhibitors,[8] it requires several steps to modify the acylated product for analysis, multiple washing steps, and requires specific specialized reaction components. The rapid advancement of studies of ghrelin acylation by GOAT and identification of GOAT inhibitors will be greatly aided by development of a "mix and measure" assay for GOAT activity compatible with high-throughput screening.

To our knowledge, the GOAT activity assay described above is the first real-time GOAT activity assay, requiring no post-reaction workup or developing steps prior to analysis. GOAT-catalyzed acylation of the ghrelin peptide is detected by an increase in fluorescence of acrylodan. Non-specific increases in fluorescence, which presumably arise from the partitioning of the peptide into the hydrophobic microsomal fraction, can be minimized by using low peptide concentrations. A negative control reaction (such as no acyl donor, using empty vector microsomal fraction, or using an inactive GOAT mutant variant) should be run in parallel for each set of reactions. In addition, the optimal volume of microsomal fraction may vary between different preparations of enzyme, so a titration of microsomal fraction protein should be performed for each new preparation of enzyme. As expression and purification protocols for GOAT improve, standardization across enzyme preparations should address this current limitation.

The loss of fluorescence enhancement in the presence of a high concentration of a known GOAT inhibitor (Figure 5.3) indicated this assay could be used to screen a library of small

molecules for novel GOAT inhibitors. The NIH Clinical Collection library was screened using this assay, with compounds tested at an initial concentration of 100 μM. Of the 700 compounds of this library, 10.3% inhibited hGOAT by at least 10-fold as measured by the fluorescence enhancement in the presence of the test compound compared to the fluorescence enhancement in the presence of the DMSO vehicle. Though this is a relatively high hit rate for a library screen, the high concentration for library compounds the primary screen was chosen to be inclusive given the overall lack of known GOAT inhibitors. In subsequent library screening studies, stringency can easily be increased by lowering the candidate compound concentration in the GOAT activity assay.

Looking forward towards development of new GOAT inhibitors, it is important to note that the NCC library consists of compounds that have already progressed to clinical trials as potential therapeutics. As such, the bioavailability of many of these compounds has already undergone some level of optimization. However, as these compounds were developed to target a wide range of diseases and disorders, they were likely optimized for activity against a molecular target other than GOAT. Therefore, it is possible that GOAT inhibitors discovered out of this library may not exhibit selectivity toward the enzyme in cell- or animal-based studies. However, the discovery of compounds from this library that do inhibit GOAT – even if only at relatively high concentrations – may help to explain or predict potential side effects of these drugs when taken for a condition unrelated to ghrelin signaling. The CDDO-based small molecule hGOAT inhibitors[10] described in Chapter 3 are an example of this, as they have shown effects consistent with altered ghrelin signaling such as improved glucose tolerance and reduced fat deposition,[23-26] despite not developed to target any part of the ghrelin-GOAT system.

The fluorescence-based GOAT activity assay described here is the first that detects acylation of ghrelin in real time. The ability to apply this assay to a high-throughput format will

accelerate the identification of new GOAT inhibitors. Although the ability to thoroughly characterize inhibitors (including measurement of IC_{50} values and determining reversibility) using this assay has not been established, this format can be used to quickly eliminate non-inhibiting compounds when analyzing structure-activity parameters of new inhibitor classes. The rapid identification of new small molecule GOAT inhibitors will accelerate the development of compounds that inhibit ghrelin signaling in biological contexts.

5.4 Materials and methods

Preparation of acrylodan labeled C4 substrate

The ghrelin peptide substrate GSSCLS-NH_2 was purchased from Sigma-Aldrich in Pepscreen format. The peptide concentration was determined by reaction of free cysteine thiol with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and measurement of absorbance at 412 nm, using ε = 14,150 $M^{-1}cm^{-1}$.[27] Concentration of acrylodan was measured by absorbance at 393 nm in methanol, using ε = 18,483 M^{-1} cm^{-1}. Labeling reactions contained 300 μM peptide and 500 μM acrylodan in 50 mM HEPES pH 7.0 in 1:1 acetonitrile : H_2O, and were vortexed for 18 h at room temperature in the dark.

The acrylodan labeled peptide was purified by HPLC with a C18 reverse phase column (Zorbax Eclipse XBD column, 9.4 x 250 mm) using a gradient of 2% acetonitrile in 0.05% TFA to 100% acetonitrile over 30 min followed by 100% acetonitrile for 10 min. Labeled peptides were monitored by absorbance at 360 nm, and eluted at a retention time of approximately 14 min. Collected fractions containing labeled peptide were dried under vacuum for 10 hr at room temperature in the dark, and resuspended in 50% acetonitrile in H_2O. Acrylodan labeling was confirmed by MALDI using α-cyano-4-hydroxycinnamic acid (CHCA) matrix using GSSCLS-$(PEG)_2$-K-biotin with a theoretical mass of 1089.5 Da as an external standard for calibration. The concentration of labeled peptide was determined by absorbance at 360 nm in water, using ε = 13,300 M^{-1} cm^{-1}.[17]

Expression of hGOAT enzyme

Expression of hGOAT was performed using baculovirus encoding the hGOAT gene with a multiplicity of infection (MOI) of 5 virus pfu/cell. For a 50 mL expression culture, 150 mL sterile

culture flasks were seeded with 1.0 x 10^8 Sf9 cells and P2 virus in 1:1 Sf900 : Insectagro media (Thermo Fisher and Corning, respectively), in a total volume of 50 mL. Expression cultures were incubated for 40 hr at 28 °C with shaking at 150 rpm. Following incubation, expression cultures were harvested by centrifugation (500 x *g*, 5 min, 4 °C), and the cell pellet was resuspended in 2.5 mL of lysis buffer containing 50 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM NaEDTA, 1 mM DTT, 0.01 μg/mL pepstatin A, 10 μM bis-4- nitrophenylphosphate, and 1 mini Roche protease inhibitor cocktail tablet per 10 mL of buffer. The cells were then lysed and homogenized with a Dounce homogenizer on ice, using 20 strokes with a small pestle and 20 strokes with a large pestle. Intact cells and debris were removed by centrifugation (3,000 x *g*, 5 min, 4 °C). The microsomal fraction was then isolated by centrifuging the supernatant (100,000 x *g*, 1 h, 4 °C). The microsomal fraction pellet was solubilized in 50 mM HEPES pH 7.0 (4 μL/mg of pellet), and was stored in 100 μL aliquots at -80 °C.

Fluorescent plate reader library screen

Reactions contained 10 μL (~50 μg, as determined by Bradford assay) microsomal fraction containing hGOAT, 1 μM MAFP, 100 μM test compound or equal volume of DMSO (1 μL), 500 μM octanoyl CoA, and 0.25 μM $GSSC_{AcDan}LS$ substrate in 50 mM HEPES (pH 7.0) in a total volume of 100 μL in 96-well black low-adhesion polystyrene plates (Corning 3650). The microsomal fraction was homogenized by passing through an 18-gauge needle 10 times, and was preincubated with MAFP and the test compound in reaction buffer for 30 min at room temperature in the 96-well plate before reactions were initiated with the addition of octanoyl CoA and peptide substrate using a multichannel pipettor. Immediately following the addition of the peptide

substrate, the fluorescence of each reaction was measured (λ_{ex} 360 nm, λ_{em} 485 nm) on a fluorescent plate reader (BioTek Synergy H1 Hybrid Reader) every 30 minutes for 3 hours.

GOAT activity was determined by calculating the percent change in fluorescence for each time point measured using equation 1:

$$(1) \qquad \% \text{ change} = \frac{Fl - Fl_{t0}}{Fl_{t0}}$$

where Fl is the fluorescence measured at each time point, and Fl_{t0} is the fluorescence at time 0. Inhibitory activity was determined for each compound by calculating the fold inhibition relative to DMSO controls using equation 2:

$$(2) \qquad \text{Fold inhibition} = \frac{\text{average \% change at 3 h of DMSO controls}}{\text{\% change at 3 h in presence of compound}}$$

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Chapter 6: Conclusions and future directions

Ghrelin signaling is an intriguing and important pathway with implications in diseases ranging from type II diabetes to addiction.[1-5] Despite extensive research into ghrelin's physiological roles since its discovery,[6] many aspects of ghrelin signaling remain unclear including the octanoylation of ghrelin by GOAT. The mechanism of GOAT catalysis is unknown, as are the specific residues involved in substrate binding and catalysis. Given the importance of fully understanding ghrelin signaling, as well as the unique nature of *O*-octanoylation, our work in the Hougland laboratory aims to determine the mechanism of ghrelin acylation by GOAT, discover how GOAT activity can be modulated through inhibition and/or agonism, and develop new tools to study GOAT activity. The work in this thesis has made progress toward each of those goals: several residues within hGOAT have been shown to be essential for ghrelin acylation activity, a new class of small molecule hGOAT inhibitors has been discovered, and modifications to our *in vitro* GOAT activity assay have been made which improve product stability and enable high-throughput screening.

6.1 Future inhibitor discovery and development

We have described a new class of hGOAT inhibitors which act through the covalent modification of a cysteine residue within the enzyme.[7] These CDDO derivatives and associated compounds are only the second class of small molecule GOAT inhibitors to be reported in the literature, and the first inhibitors which do not feature a lipid chain mimicking the octanoyl group of acylated ghrelin.[8] These new inhibitors, featuring a synthetic triterpenoid scaffold and an

electrophilic α-cyanoenone, inhibit hGOAT in our *in vitro* enzyme activity assay with IC_{50} values in the low micromolar range.

While the discovery of these cysteine-modifying hGOAT inhibitors provided valuable information regarding the possibility of a cysteine residue involved in catalysis, their ability to inhibit GOAT in cell-based assays has not been conclusively established. CDDO derivatives including those that inhibit hGOAT have been shown to have several other biological targets,[9] and a clinical trial evaluating CDDO-Me as a treatment for chronic kidney disease was terminated due to deleterious cardiovascular events.[10] Therefore, these would not be selective for GOAT without further optimization. Subsequent evaluation of patients taking part in the CDDO-Me clinical trial revealed that specific risk factors for heart failure predicted the deleterious cardiovascular effects leading to termination of the study, and thus should be used as exclusion criteria in future clinical trials.[11] This study and others evaluating CDDO derivatives have reported side effects consistent with altered ghrelin signaling,[10, 12-14] establishing that these drugs may still be useful therapeutics targeting GOAT, as long as the effects of other targets can be accounted for.

While currently the best characterized example of a small molecule GOAT inhibitor identified from a library screen in our work, CDDO-Im was just one of several hits from the initial screen of the Diversity Set IV library. The structure-activity parameters of another small molecule from this library are currently being analyzed in our laboratory in collaboration with the Chisholm laboratory. The initial hit compound has an IC_{50} in the mid-micromolar range in our *in vitro* hGOAT activity assay, and following a full structure-activity analysis it will be exciting to determine the ability of this new class of inhibitors to inhibit ghrelin acylation in a cellular context.

In separate studies, other researchers in the Hougland laboratory are working to develop new peptide-based hGOAT inhibitors. Previous work in our lab determined structural elements of

ghrelin's N-terminal residues important for recognition and modification by hGOAT.[15, 16] Expanding upon studies establishing that mimics of acylated ghrelin are potent GOAT inhibitors,[17, 18] our lab has developed a panel of non-acylated peptides based on ghrelin's N-terminus which inhibit hGOAT in our *in vitro* enzyme activity assay in the nanomolar range. Structure-activity analysis of these inhibitors has established functional groups involved in binding to the enzyme.

One of the biggest challenges in discovering and developing GOAT inhibitors lies in inability to purify the enzyme in an active form.[19] Assays studying GOAT activity and inhibition are either cell-based or rely on enriched, but unpurified, microsomal fraction. As described in Chapters 2 and 5, the incorporation of microsomal fraction into GOAT activity assays inherently introduces additional components (liposomes and other membrane-associated proteins), that make the application of GOAT assays to a high-throughput format difficult, preventing screens of large libraries of small molecules for GOAT inhibitors. Ultimately, improved protocols for purification of integral membrane proteins may enable the purification of GOAT, enabling the development of better activity assays and possibly the determination of its structure within the ER membrane. An accurate, high-resolution picture of the substrate binding sites and active site of GOAT will provide valuable information about contact sites that may be exploited in the design or optimization of potent, selective small molecule inhibitors.

6.2 Developing tools to efficiently investigate GOAT enzymatic activity

Biochemical studies of GOAT have been limited since its discovery by the lack of tools for efficiently studying enzyme activity. Low levels of product conversion and assays requiring extensive workup have prevented extensive enzymatic characterization or their application to high-

throughput screening for inhibitors. We have made several improvements to the *in vitro* fluorescence-based GOAT assay which resolve both of these limitations.

Alkyl fluorophosphonates such as MAFP inhibit non-specific esterases present in enriched microsomal fraction used as the GOAT enzyme source in several reported GOAT activity assays.[15, 17, 19, 20] These esterases degrade the acylated ghrelin product of the GOAT reaction to the unacylated peptide and inhibiting the esterases with MAFP allows for much greater product conversion and longer reaction times.[21] MAFP appears to inhibit a range of ghrelin-active esterases, as it effectively protects acyl ghrelin and acyl ghrelin mimetic peptides from degradation in microsomal fraction from insect cells, in HEK293FT cell lysate, and in rat blood plasma.

We have also developed a GOAT activity assay based on the fluorescence enhancement of acrylodan-labeled ghrelin peptides upon acylation. This readout does not require separation of reaction products, so GOAT activity can be analyzed in real time on a fluorescent plate reader. We have shown that fluorescence enhancement is specific to ghrelin acylation, as the enhancement is blocked by omitting octanoyl CoA or enzyme, and by treatment with both peptide-based product mimetic and small molecule inhibitors. This new assay will allow for more rapid identification of novel small molecule inhibitors of hGOAT through high-throughput screening of libraries.

Our lab is currently developing additional tools to study hGOAT-catalyzed ghrelin acylation. The advancement of any GOAT inhibitor depends upon proven activity in cells. Toward this end, a cell line is currently being developed to efficiently study the effects of potential GOAT inhibitors in a biologically relevant context. A prostate cancer cell line expressing preproghrelin and GOAT was shown to increase proliferation in response to treatment with ghrelin.[22-24] Ghrelin-dependent proliferation of these cells may be an alternative readout for GOAT activity, which would provide an orthogonal screening platform for GOAT inhibition.

6.3 Determination of GOAT substrate binding sites and catalytic mechanism

As described in Chapter 4, locating the substrate binding sites and determining the mechanisms for protein acylation remains an unmatched challenge for all members of the MBOAT superfamily despite recent successes in experimentally determining GOAT and Hhat membrane topologies.[25-27] Through mutational analysis of hGOAT, we have determined several residues that are required for ghrelin octanoylation activity. Some of these residues are candidates for the general base we have proposed to be involved in activation of ghrelin's serine 3 hydroxyl for nucleophilic attack. There are also multiple cysteine residues that appear to be important for activity, as mutation of cysteine 235 individually to either alanine or serine and mutation of the five cysteine residues within transmembrane helices 1 – 4 of hGOAT to serine collectively results in complete loss of enzyme activity. Mutant variants of hGOAT in which individual cysteines are mutated to serine are currently being developed, and will help to determine specific residues which may be involved in catalysis.

Moving forward, a number of avenues are being pursued to gain a structural understanding of GOAT-catalyzed ghrelin acylation. In a collaboration between our lab and the Nangia laboratory of the Biomedical and Chemical Engineering Department at Syracuse University, computational modeling coupled with mutational analysis has determined a number of residues within hGOAT that are predicted to be important for ghrelin acylation. These studies are beginning to define domains within GOAT that are required for substrate binding, enzyme stability, and catalysis.

In addition to mutational analysis based on individual residues, constructs consisting of a conserved loop domain within hGOAT are also being expressed for biochemical and structural characterization. A large cytosolic loop between transmembrane helices 7 and 8 is highly

conserved between GOAT and Hhat, and contains the conserved and functionally essential N307 residue.[25-27] Structural and biochemical characterization of this domain on its own will illuminate its role in GOAT activity, and provide the first information regarding subdomain conformation within GOAT.

Collectively, these studies will provide a comprehensive view of ghrelin acylation by GOAT. Mutational analysis alone reveals residues which are required for enzyme activity, but in conjunction with computational analyses, biophysical and structural studies, and bioinformatics analysis across species, our lab may elucidate which of those functionally essential residues are involved in enzyme stability, substrate binding, and catalysis.

6.4 Summary and future direction

We have made several advances in this work toward the study of ghrelin acylation by hGOAT, including the discovery of a new class of small molecule inhibitors, the determination of residues which are required for activity, and developments to our assay which improve sensitivity and product stability and enable real-time monitoring of GOAT activity. As each of these projects continue forward, progress in one area will inherently inform and help progress the others. For instance, one ultimate goal of our lab's work is to determine the mechanism by which GOAT catalyzes ghrelin octanoylation. When the residues involved in binding to either the ghrelin peptide or octanoyl CoA are determined, we may be better able to rationally design or optimize GOAT inhibitors by exploiting these contacts. In addition, discoveries made in other MBOAT family members – especially those that also acylate protein substrates, Hhat and PORCN – may help us better understand those aspects in GOAT. Given the remarkable similarity between the topologies

of Hhat and GOAT within the ER membrane,[25-27] it seems likely that these enzymes employ similar mechanisms and are susceptible to similar inhibitors.

Given ghrelin's role in an ever-growing number of physiological processes and in diseases such as diabetes and obesity, studies such as these are imperative to understanding the entire picture of ghrelin activation and signaling within the body. These studies will also aid in determining if and how pharmaceutical modulation of ghrelin signaling *in vivo* is a therapeutic avenue for the treatment of these and other diseases. Ghrelin's role in the body goes far beyond that of a hunger-inducing ligand for the GHSR1a receptor. The "traditional" view of ghrelin processing requires octanoylation by GOAT in the ER, and subsequent binding to GHSR1a to induce its biological effects. Yet it is now known that unacylated ghrelin exerts its own biological effects, prompting the questions: does unacylated ghrelin have its own unique receptor? And is the hydrolysis of ghrelin to unacylated ghrelin independently regulated through a ghrelin esterase? Several esterases have demonstrated *in vitro* activity against ghrelin[28-31] but it is not known whether ghrelin is specifically regulated through these enzymes *in vivo*. Another related question is whether ghrelin acylation/deacylation is a dynamic process in the body, similar to the reversible nature of palmitoylation.[32] A recent study reported the localization of active GOAT within the plasma membrane of mouse bone marrow adipocytes, suggesting that ghrelin can be acylated directly at its target cells.[33] Replication of this study in bone marrow adipocytes and other tissues would lend weight to the idea that ghrelin acylation is dynamic, even after secretion from the cell.

With every new publication regarding ghrelin, the signaling pathway of this hormone appears to be increasingly complex with many aspects remaining undefined. Determination of a complete, accurate picture of the roles played by ghrelin in the body depends on the development of tools to accurately study ghrelin expression, acylation, deacylation, and interactions with other

biomolecules. Small molecules GOAT inhibitors such as the CDDO derivatives may serve as clinically useful therapeutics for diseases such as diabetes, but they will also be used as pharmacological tools to temporally control GOAT activity when studying ghrelin signaling in cell or animal models. Improvements to GOAT activity assays described in this work will also aid in the discovery of new, more potent, and more selective GOAT inhibitors, and may also be applied to studying other aspects of the ghrelin signaling pathway, such as esterase-catalyzed deacylation of ghrelin.

Mutational analysis of hGOAT, structure-activity analysis of hGOAT inhibitors, and development of *in vitro* enzyme activity assays described in this work contribute to the ultimate goal of understanding how GOAT catalyzes the octanoylation of ghrelin, an essential and largely enigmatic step in ghrelin processing. Future work in the Hougland laboratory and others studying GOAT may provide answers for the many questions still surrounding this enzyme and the other aspects of the ghrelin signaling pathway.

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Education

Ph. D.	Department of Chemistry Syracuse University, Syracuse, NY	*Summer 2017 (anticipated)*
M. Phil.	Department of Chemistry Syracuse University, Syracuse, NY	2014
B. S.	Department of Chemistry St. John's University, Queens, NY	2012

Research Experience

2012-present	**Enzymology and inhibition of ghrelin acylation by ghrelin *O*-acyltransferase** *Doctoral Dissertation, Syracuse University* Advisor: Prof. James L. Hougland
2010-2012	**Investigation of interfacial free energy of aliphatic and aromatic surfaces in aqueous solutions by atomic force microscopy** *Undergraduate Investigator, St. John's University* Advisor: Prof. Joseph M. Serafin

Awards and Fellowships

2016	ACS Women Chemists Committee Travel Award, *Eli Lily & Company*
2014	Nominated for Women in Science and Engineering Future Professionals Program, *Syracuse University*
2012	Graduate Assistance in Areas of National Need Fellowship, *Syracuse University*
2011	St. John's College Gold Key Recipient, *St. John's University*

Publications

K. R. McGovern-Gooch, N. S. Mahajani, A. Garagozzo, A. J. Schramm, L. G. Hannah, M. A. Sieburg, J. D. Chisholm, and J. L. Hougland
"Synthetic triterpenoid inhibition of human ghrelin *O*-acyltransferase: Involvement of a functionally required cysteine provides mechanistic insight into ghrelin acylation."
Biochemistry. **2017**, 56(7):919-931

K. R. McGovern-Gooch, T. Rodrigues, J. E. Darling, M. A. Sieburg, A. Abizaid, and J. L. Hougland
"Ghrelin octanoylation is completely stabilized in biological samples by alkyl fluorophosphonates."
Endocrinology. **2016,** 157(11):4330-4338

K. R. McGovern, J. E. Darling, and J. L. Hougland
"Progress in small molecule and biologic therapeutics targeting ghrelin signaling."
Mini Rev Med Chem. **2016,** 16(6):465-480

K. R. McGovern-Gooch, J. E. Darling, J. L. Hougland
"A fluorescence-based high throughput assay to rapidly identify small molecule inhibitors of ghrelin acylation by ghrelin *O*-acyltransferase"
In preparation

K. R. McGovern-Gooch, M. B. Campaña, R. L. Loftus, and J. L. Hougland
"Toward defining the ghrelin *O*-acyltransferase active site: Determination of functionally essential residues within hGOAT"
In preparation

Teaching Experience

Spring 2017 Spring 2016	**Teaching Assistant,** Organic Chemistry II Recitation, Syracuse University *Led students through organic chemistry problems supplementing the course* Advisor: Prof. James Kallmerten
Fall 2015	**Teaching Assistant,** Organic Chemistry I Recitation, Syracuse University *Led students through organic chemistry problems supplementing the course* Advisor: Prof. James L. Hougland

2014-2017	**Undergraduate Research Mentor,** Biochemistry, Syracuse University *Mentored four undergraduate students in biochemical laboratory techniques* Advisor: Prof. James L. Hougland
Spring 2015 Fall 2013	**Teaching Assistant,** General Chemistry Laboratory, Syracuse University *Instructed laboratory experiments designed to reinforce and supplement the course* Advisor: Gary Bonomo

Poster Presentations

August 2016	"Ghrelin acylation by ghrelin *O*-acyltransferase: Enzyme mutagenesis studies and inhibitor library screening" **Kayleigh R. McGovern**, Nivedita Mahajani, Anthony J. Schramm, Ariana Garagozzo, John Chisholm, and James L. Hougland 252nd American Chemical Society National Meeting Philadelphia, PA
July 2015	"Ghrelin acylation by human ghrelin O-acyltransferase: Enzyme mutagenesis studies and inhibitor library screening" **Kayleigh R. McGovern**, Anthony J. Schramm, Ariana Garagozzo, and James L. Hougland FASEB Science Research Conference for Protein Lipidation, Signaling and Membrane Domains Saxtons River, VT
June 2015	"Investigating human ghrelin *O*-acyltransferase: Functional studies and inhibitor screening" **Kayleigh R. McGovern**, Anthony J. Schramm, Ariana Garagozzo, and James L. Hougland American Chemical Society Northeast Regional Meeting Ithaca, NY
May 2014	"Defining the catalytic machinery of hGOAT" **Kayleigh R. McGovern**, Rosemary J. Loftus, and James L. Hougland Chemistry Graduate Student Symposium (SUNY-Buffalo), Buffalo, NY